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Guidelines for Preparing Environmental and Waste Samples for Mutagenicity (Ames) Testing Interim Procedures and Panel Meeting Proceedings

Life Systems, Inc., Cleveland, OH

Prepared for

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16. ABSTRACT

This User's Guide presents review papers and interim protocols for the preparation of waste and environmental samples for testing with the Salmonella reverse-mutation assay (Ames test). Sample types addressed include air, drinking water (processed), environmental and wastewaters, nonaqueous liquid wastes, soils and sediments, and waste solids. Draft procedures were reviewed and revised by an expert panel of chemists, biologists, and toxicologists during meetings held in Palo Alto, California, July 23-25, 1984. By consensus, the resulting protocols were recommended by the expert panel to; (1) serve as best available interim methods for sample preparation, and (2) receive the additional laboratory characterization required for establishment as reference methods.

This User's Guide is intended to partially fill the existing gap in the scientific literature for standardized generally accepted methods to prepare environmental or waste samples for mutagenicity testing.

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by

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Cleveland, Ohio 44122

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The efforts of all these individuals contributed to the success of the Panel Meeting and the development of the media-specific sample preparation protocols for short-term mutagenicity testing which appear in this document.

SECTION 1

EXECUTIVE SUMMARY

INTRODUCTION

This publication is a compilation of the review papers and sample preparation protocols presented, discussed and revised during the meeting entitled, "Mutagenicity Sample Preparation Protocols Panel Meeting," held July 23-25, 1984, in Palo Alto, California. The Panel Meeting was organized and conducted to facilitate development of sample preparation protocols for mutagenicity testing of six media: air, drinking water, nonaqueous liquid wastes, soils and sediments, waste solids and wastewater.

Background

The available interim Ames testing procedures, under evaluation in a multi-laboratory testing program sponsored by the U.S. Environmental Protection Agency (EPA), do not address sample preparation for wastes and environmental materials. Further, there are no generally accepted or standard methods for preparing these types of samples. Both EPA's Environmental Monitoring Systems Laboratory-Las Vegas, NV (EMSL-LV), and the U.S. Army Medical Bioengineering Research and Development Laboratory (MBRDL) need procedures for the preparation of wastes and environmental samples for mutagenicity testing to ensure comparability of test results for scientific evaluation and for potential hazardous waste site enforcement actions and litigation.

Objectives

The overall objective of the project was to develop sample preparation protocols for mutagenicity testing of wastes and environmental samples from six media: air, drinking water, nonaqueous liquid wastes, soils and sediments, waste solids and wastewater.

The following objectives were established for the Mutagenicity Sample Preparation Protocols Panel Meeting:

1. Evaluate the adequacy and validity of the selected and reviewed sample preparation protocol in each medium.
2. Prepare revised sample preparation protocols in accordance with review comments and recommendations from the Panel Meeting participants.
3. Present a scientific basis for any disagreements or unresolvable issues.

4. Make recommendations for additional research to support further development of medium-specific sample preparation protocols.

Approach

Prior to the Panel Meeting, representatives from industry, academia and government were selected and assigned to one of six media Workgroups for the Panel Meeting on the basis of past research or demonstrated experience in application of sample preparation methods for mutagenicity testing. (See Appendix 1 for the List of Participants). Workgroup Leaders reviewed available literature, consulted colleagues, selected the best available sample preparation protocol for their medium and prepared a Review Paper to support their selection. Members of each Workgroup provided written comments on the Review Paper and the selected Protocol. Workgroup Leaders responded to these comments prior to the Panel Meeting and completed revised Review Papers and Protocols.

The three-day Panel Meeting consisted of several General Sessions and Workgroup Sessions. The Workgroup Leaders presented the Review Papers to all Meeting participants at the General Sessions, and group discussions followed (see Appendix 2 for the Agenda). Issues identified during the General Sessions and any unresolved written review comments were addressed during the Workgroup Sessions. Interaction among Workgroups was accomplished by presentation of brief progress reports from Workgroup Leaders at the General Sessions. In several cases, hybridization of available protocols was necessary to resolve issues, develop media-specific protocols and attain consensus among Workgroup members. Each Workgroup completed its recommended Protocol and a separate Workgroup Report which summarized the key issues pertinent to that medium. The recommended Protocols and rationales for revisions made by the Workgroups were presented to all Panel Meeting participants. A consensus among meeting participants was achieved on all recommended Protocols prior to the conclusion of the Panel Meeting.

GENERAL ISSUES AND RESOLUTIONS

This section summarizes the major issues identified at the General Sessions of the Panel Meeting and the resolutions proposed by the Workgroups. For additional details, refer to the Review Papers, Workgroup Summary Reports and Protocols which appear in subsequent sections of this report.

Media Definitions

Sample preparation media were defined by the participants to assist laboratories in selecting the appropriate protocol for a specific sample. The definitions were intended to encompass the continuum of wastes and environmental materials, to reduce overlap between individual medium definitions and to reflect the scope of the protocols. The following media definitions were developed:

- Air Particulate Matter: Particulates collected from the air which are 20 microns or less in diameter.

- Drinking Water: Water intended for human consumption.

- Environmental Waters and Wastewaters: Waters containing less than 50% suspended solids by weight, including water from lakes, ponds, lagoons, estuaries, rivers, streams, effluents and groundwater.

- Nonaqueous Liquid Wastes: Liquids whose major component is not water. These materials range from soluble organic liquids (e.g., acetone) to insoluble organic liquids (e.g., dichloromethane) and liquids such as light to heavy oils.

- Soil and Sediments: The unconsolidated material on the earth's surface capable of supporting plant growth and soil material deposited and remaining in an aquatic environment, respectively.

- Waste Solids: Waste or complex mixtures, less than 50% water, having relative firmness, coherence of particles or persistence of form as matter that is not liquid or gaseous at 25 C. This includes tarry material that is sticky or viscous, such as coal tar, adhesive waste, sludge, airborne particulates (e.g., re-entrained particles blown from a waste site) and solids partitioned from wastewater.

Examples and further elaboration of these definitions are contained in each Protocol.

Scope and Limitations of Protocols

Discussions to determine scope and limitations provided the following key points, requirements and resolutions for the protocols:

- They must provide end products that meet minimal input requirements for Ames mutagenicity testing.

- They should be quantitative to enable evaluation of any dose-response relationships.

- They must be developed with consideration of the ultimate user (i.e., comprehensive, stepwise procedures designed for the inexperienced user).

- They will be used to determine whether samples are mutagenically active or non-active (i.e., positive or negative types of determinations).

- They will be applied to screen large numbers of complex mixtures within a variety of media.

- Development and standardization of the protocols will be a dynamic process that involves establishing interim procedures that are optimized and modified as a result of validation testing.

Toxicity and Fractionation

A variety of criteria are used by testing laboratories to determine toxicity during the Ames test and when additional sample preparation (i.e., fractionation) is required to separate toxic components from potential mutagenic components of a complex mixture. However, no uniform criteria are available or accepted. The following criteria were proposed and accepted by Panel Meeting participants as the appropriate "conceptual basis" for determining lack of toxicity during the Ames test:

- A dose level of $\geq$ 1 mg/plate of residue organic matter is achieved; and
- The background lawn is not affected; and
- Spontaneous revertants are countable and comparable to control plates.

When these criteria are met, the material is not toxic, and fractionation may not be required. Establishment of the 1 mg/plate level as the definition of toxicity and the trigger for fractionation was arbitrary but considered reasonable by the Panel Meeting participants. It was recommended that toxicity determinations be performed with the entire set of bacterial strains (with and without enzymatic activation) being used. Concerns expressed regarding the above criteria included the following:

- Possible misinterpretation by the user that testing at the $\geq$ 1 mg/plate level is required to prove non-mutagenicity.

- Limited applicability to the Air Protocol because the amount of material collected is often insufficient to allow dosing of $\geq$ 1 mg/plate with multiple strains and replicate plates.

Although declining to recommend using a high-performance liquid chromatography (HPLC) technique in lieu of the acid-base extraction procedure, the Panel Meeting participants identified it as a potential sample preparation technique if toxicity is detected. The available techniques to reduce or separate toxic activity from potentially mutagenic components of a mixture included:

1. Liquid-liquid acid-base extraction fractionation
2. Thin-layer chromatography (TLC)
3. Florosil chromatography
4. Low-pressure chromatography
5. Dilution of neat material with dimethyl sulfoxide (DMSO)
6. HPLC fractionation

The effect on reactive compounds and potential alteration of mutagenic activity of the acid-base extraction procedure were a major concern. The TLC method was not recommended because of compound loss due to reactivity of the chemical binder. The Florisil and low-pressure chromatographic methods were considered to be research methods.

The HPLC technique was viewed as the most recent technology that may be a viable alternative to acid-base extractions. However, the following issues were raised concerning HPLC:

- Time required to run step gradient
- Ability to screen large numbers of samples
- Reliability and column life
- Equipment down-time
- Operating costs
- Training level of operator
- Incompatibility of certain compounds to HPLC

The Panel Meeting participants concluded that both the acid-base extraction and HPLC techniques should be identified in the protocols and the choice of fractionation methods should be at the discretion of the user. A Review and Protocol for the HPLC technique are provided in Appendix 3.

PROTOCOL-SPECIFIC ISSUES AND RESOLUTIONS

The following summaries present the major protocol-specific issues voiced at the Panel Meeting with the resolutions achieved.

Air Particulates Protocol

The Air Protocol is restricted to sample preparation of particulate organic material (POM) and excludes vapor phase and volatile organic compounds. Mutagenicity testing of volatiles and vapor phase organics is a research technique rather than a routine screening or monitoring method appropriate for current needs of the EPA and the Army. The definition of "air particulates," presented earlier, clarifies the scope of the Protocol.

The Protocol focuses on preparation of collected particulate samples, rather than addressing detailed methods for sample collection, sampling strategy and equipment. The available sampling techniques and equipment (e.g., Hi-Vol sampling of ambient air) are presumably capable of providing a sufficient quantity of POM for sample preparation via the Protocol and of yielding quantities that meet the input requirements of the Ames mutagenicity assay. Compositing samples from multiple Hi-Vol samplers or using scaled-up samplers (i.e., Ultra Hi-Vol Sampler) make it possible to collect a sufficient quantity of POM where mutagenicity bioassays, gravimetric determinations, chemical analyses or sample archiving are planned. Generalized procedures for particulate collection are inappropriate, since selection and implementation of appropriate methods are source dependent.

The Soxhlet technique is recommended as the primary extraction method, but sonication is included as an alternative. Side-by-side validation studies for evaluation of these alternative extraction techniques are required to determine their equivalency.

Several available options for extraction solvents are included in the Protocol to give the investigator flexibility. These include using a single solvent (cyclohexane or dichloromethane), sequential solvent extraction (cyclohexane, then dichloromethane) or solvent mixtures (cyclohexane, dichloromethane and methanol).

The Kuderna-Danish (K-D) apparatus is preferable to the rotary evaporator for solvent volume reduction if extract components are thermally stable. The rotary evaporator is suitable for large volume reduction applications, but tends to lose material as the extract approaches dryness. Both methods are included in the Protocol to allow flexibility. Additional research is recommended to evaluate the need for solvent exchange.

Drinking Water Protocol

The Drinking Water Protocol is limited to "residue organics" that can be absorbed on XAD resins and recovered by solvent elution techniques. The inability of the Protocol to address highly polar, ionic species and highly volatile and low molecular weight organic compounds that are not absorbed or recovered from the collection apparatus is emphasized. The Protocol is applicable for all types of finished drinking water. The type of disinfection treatment (if any) is not considered to be a critical issue. A glass wool prefilter, a Celite(a) column and a bacterial filter are incorporated when waters contain more than 5% solids, are not disinfected or contain more than 20 parts/million (ppm) total organic carbon.

Detailed methods for cleaning the XAD resins are presented to ensure that a uniform approach is implemented for determinations of resin purity and to eliminate potential for contamination. Other quality controls include blanks, spiking of samples, controls, duplicates and frequency of sampling.

Additional research is needed to evaluate using additional columns such as cation and anion exchange resins to recover highly polar or ionic species from dilute aqueous solutions. Recovery studies using a variety of organic compounds with a wide range of polarities and solubilities are recommended to determine the efficiency of this system. Radiolabeled compounds and a variety of surrogate chemicals should be used to evaluate irreversible binding to resins and to quantify compound loss in the collection apparatus.

Environmental Waters and Wastewater Protocol

This medium is multi-phasic and covers a wide range of constituents, including aqueous and nonaqueous liquids and dissolved and suspended solids. The Protocol is limited to solvent-extractable organic compounds; however, not all compounds will be recoverable and/or stable under the Protocol's methods. An overall scheme was developed and incorporated in the Protocol to link the variety of components of this medium to the other Protocols.

(a) Registered trademark.

Each discrete phase of this multi-phase medium is collected, quantified, separated and concentrated or extracted during sample preparation. Gravity separation (24-hr duration) of the sample into three distinct phases initiates the process. Standard methods for determinating percent suspended solids in the aqueous liquids are included in the Protocol. The Protocol for Drinking Water is used to prepare aqueous liquids with less than 5% solids. Aqueous liquids with more than 5% solids are prepared by one of the following methods: (1) liquid-liquid extraction, concentration and solvent exchange; (2) separation by filtration or centrification, with the liquids being processed according to the Drinking Water Protocol and the solids according to the Waste Solids Protocol. The solid sediments from the initial 24-hr gravity separation are processed according to the Protocol for Waste Solids. Nonaqueous liquid wastes partitioned from the samples are processed via that Protocol.

Nonaqueous Liquid Wastes Protocol

Nonaqueous liquid wastes were defined to include compounds that range from water-soluble organic liquids to immiscible oils. Only a limited amount of data are available on the applicability of this Protocol to compounds other than oils or petroleum products. This medium differs from the other media because materials are often concentrated. The Protocol incorporates dilution steps in addition to the concentration techniques used in the other media Protocols. This Protocol is unique because of the opportunity to test neat material or neat material diluted with DMSO without losing polar compounds. This medium enables analyses with complete samples rather than with compounds collected on an absorbent or extracted with a solvent. Testing using neat samples or DMSO suspensions is recommended as an initial test.

Procedures for solvent extraction are also included in this Protocol as a second step. Dichloromethane is recommended as the choice of solvent rather than diethyl ether for several reasons. Dichloromethane promotes uniformity with the other Protocols' solvent selection, is more readily obtainable in a high purity form and reduces potential safety problems. Identification of the optimum solvent(s) remains a research question that should be addressed by extraction efficiency studies comparing various solvents, mixtures or sequences.

No specific time requirement for storage is specified due to the lack of available scientific data on degradation; however, it is recommended that the neat material be tested as soon as possible.

No adjustment of pH is recommended for the neat sample. In cases where fractionation is required, extraction of bases with hydrochloric acid and subsequent separation of the acid and neutral fractions are recommended to avoid destruction of mutagens. The acid-base fractionation procedure is identical to that used in the Soils and Sediments Protocol and the Waste Solids Protocol. The Workgroup made specific recommendations not to perform additional fractionation if toxicity is observed in the DMSO acid, base or neutral fractions. The acid-base fractionation procedures are not suitable for low concentrations of highly polar mutagens. The HPLC technique is an appropriate alternative to the acid-base extraction method.

The use of internal standards and the selection of appropriate compounds for spiking are other major issues. Addition of three known mutagens that would be fractionated into the acid, neutral and base solutions, respectively, is recommended prior to the concentration stage. This procedure enables determination of potential loss of mutagenicity during fractionation.

Additional quality controls, such as using a solvent-blank control and a positive control for all sample preparation techniques and subsequent testing for mutagenicity, are included. The potential for contamination of glassware due to the highly concentrated nature of the nonaqueous liquid wastes is alleviated by warning the analyst about cross-contamination and specifying rigorous glassware cleaning methods or use of disposable glassware or vials.

Soils and Sediments Protocol

Most of the technical issues regarding this medium relate to the extraction step, including solvent choice, extraction method, volume reduction and efficiency. Dichloromethane is the selected extraction solvent because of its universality and use in the other Protocols. Sequential solvent extractions or extraction with solvent mixtures are appropriate in cases where information on specific soil contaminants in the sample is available. The appropriateness of using a single solvent versus solvent sequence or mixture for preparation of soils remains a research issue.

The Soxhlet is the primary extraction method recommended because of its extensive use and acceptance. The blender and sonicator techniques are listed as alternative extraction procedures that may be faster or more efficient. Determination of the equivalency or superiority of these alternative methods for soil extraction remains a research issue. Use of the rotary evaporator is the only volume reduction method recommended.

Determining the soil sample's pH prior to extraction is recommended. Additional research to determine the effect of pH adjustment on the solvent extraction efficiency of mutagens is, however, needed. Specific research recommendations included designing studies to determine extraction efficiency for a variety of organic compounds (with different polarities or functional groups) in several soil types with pH adjustments within the range of 4 to 8. Determinations of the optimum pH and solvent(s) (single, sequence or mixture) are the primary research program needs.

Use of the acid-base neutral fractionation technique or the HPLC method is recommended to be at the discretion of the investigator. Recommendations are made to evaluate these techniques in a side-by-side experiment to identify alteration or destruction of mutagenic activity from soils and sediments. The acid-base extraction technique is the most widely accepted method; however, the HPLC method is a useful alternative procedure.

Use of an internal standard for quality control may be inappropriate, since it could obscure the analysis of the soil sample. A negative solvent control is an adequate blank, and utilization of a universal soil blank is not necessary. Furthermore, standard blank soil samples are not available.

Optimum storage conditions for soils and sediments remain presently undefined. There is an absence of quantitative data on the stability of organic pollutants in soil and sediments under a variety of storage conditions. Experiments on degradation of spiked compounds under different storage temperatures, moisture content, storage times and soil or sediment types are recommended to determine optimum conditions. Autoclaving is discussed as a technique that may improve soil stability, but resulting alteration of mutagenic activity needs to be addressed by further research.

Waste Solids Protocol

The Protocol's scope is limited to organic components of waste solids that are solvent-extractable and remain stable through the sample preparation procedure. This Protocol is based on well established analytical chemical methods that have been used extensively to determine compositions of complex organic mixtures and environmental samples. However, only limited data are available to demonstrate the efficacy of such methods to prepare waste solids samples for mutagenicity assays. Vapor phase organics and inorganic components of waste solids are intentionally not addressed in the Protocol.

Waste solids are defined as heterogeneous materials that range from sticky, viscous or tarry material to dry solid particulates. Special techniques for treatment of oily, gummy and adhesive materials, (e.g., addition of anhydrous sodium sulfate or silica gel) are specified in the Protocol. The gravity phase separation procedure (24 hr at 4 C) developed by the Environmental Waters and Wastewater Workgroup is incorporated by reference to address removal of liquids from waste solids samples.

The Waste Solids Protocol applies to waste solids samples partitioned from aqueous or non-aqueous liquids and potentially from ambient air or gaseous media. Freezing samples or adding dry ice prior to grinding for particle size reduction are considered comparable alternative pretreatment procedures. Reduction of waste solids to a maximum particle size of 2 mm or less is recommended to enhance the extraction procedure.

The extraction procedure is a key issue. The Soxhlet technique is selected as the primary method with use of a blender as an acceptable alternative. Additional research is recommended to evaluate efficiencies and equivalency. The addition of surfactants or other compounds to improve extraction efficiency is not recommended except in cases where supporting scientific data are indicative. Dichloromethane is the recommended solvent. Use of DMSO is limited due to interferences with chemical analyses and reduction in extraction efficiency. Extraction should be conducted at 4 C to reduce degradation of complex mixtures. The efficiency of specific solvents, sequences or mixtures for the extraction of waste solids remains a research issue.

The K-D apparatus is the primary method for solvent volume reduction; however, the rotary evaporator may be used to concentrate extracts when the loss of moderately volatile chemicals is not of concern. The solvent DMSO is used to redissolve an aliquot of the concentrated crude extract from the K-D apparatus. Redissolution with ethanol, acetone or other solvents may be appropriate for certain types of samples.

An overall testing scheme is presented to clarify decisions regarding the toxicity and implementation of fractionation techniques. Initial applications of crude extract material at dose levels ranging from 10 ng to 10 mg/plate are recommended for non-toxic materials. Additional sample preparation and testing are not necessary if a positive (i.e., mutagenic) non-toxic response is observed. Acid-base fractionation is recommended only in cases where toxicity is observed or characterization of specific mutagens is desired. Fractionation is optional in cases where negative, non-toxic responses result.

The Workgroup acknowledges that acid-base extraction can potentially alter sample components. However, implementation of the alternative HPLC fractionation method is not warranted due to lack of available scientific information and the intended objective of evaluating overall mutagenicity of the sample. The liquid-liquid acid-base extraction procedure is therefore recommended, after modification, to reduce potential degradation of components in complex mixtures. Modifications include (1) dissolving the sample in dichloromethane (or the extraction solvent) prior to fractionation, (2) acid separation prior to base separation and (3) performing separations at 4 C. Hydrochloric acid is preferred to sulfuric acid to reduce compound decomposition.

Storage at 4 C or less is recommended for all samples and at -20 C or less for crude and processed extracts. The recommended containers are amber glass bottles with Teflon tops or Teflon containers for aggressive compounds. Stainless steel containers are not considered desirable because of the potential for sample contamination. Additional research to address optimum storage conditions (temperature and time) and storage containers to preserve sample integrity is recommended. This includes research on the storage of raw samples, crude extracts and processed extracts.

PANEL MEETING CONSENSUS ON SAMPLE PREPARATION PROTOCOLS

Air Particulates

There was unanimous agreement among Panel Meeting participants that the Air Particulates Protocol is a proven method with an adequate data base to demonstrate applicability to preparation of air particulates for mutagenicity testing. However, the participants agreed that there is not a sufficient data base to enable evaluation of the equivalency of alternatives provided in this Protocol, such as (1) selection of solvent(s) for extraction (i.e., single solvent versus sequential use of solvents versus mixture of solvents), (2) methods for solvent volume reduction (i.e., rotary evaporation versus K-D reduction) and (3) solvent exchange steps. Validation studies comparing these alternatives are required prior to making final recommendations on solvent selection, volume reduction and solvent exchange.

Drinking Water

The Panel Meeting participants were in unanimous agreement that the Drinking Water Protocol is a well established method with a sufficient foundation of data that demonstrate the adequacy of its application to isolating residue organics from drinking water for mutagenicity testing. This consensus to recommend the Drinking Water Protocol was made with cognizance of the inherent limitation that highly polar and ionic species or highly volatile low molecular weight organics may not be recovered. The Panel Meeting participants recommended that the Protocol be subjected to validation studies by various laboratories. Additional research on the method was not considered necessary.

Environmental Waters and Wastewater

The Panel Meeting participants were in unanimous agreement that the Environmental Waters and Wastewater Protocol is a synthesized protocol that assembles portions of a number of established methods. There is a good indication that this combination of components will work together; however, a comprehensive data base demonstrating the efficacy of the hybridized combination of methods needs to be generated and evaluated.

Nonaqueous Liquid Wastes

There was unanimous agreement among Panel Meeting participants that the Nonaqueous Liquid Wastes Protocol is a synthesized protocol that assembles portions of a number of proven methods. There is a good indication that the various hybridized components will be applicable to a range of nonaqueous liquids; however, this is based on available data from testing of discrete types of oily compounds. Further studies were recommended to evaluate applicability to the variety of nonaqueous liquids and to determine the optimum solvent for separation or fractionation of these compounds.

Soils and Sediments

There was consensus among the Panel Meeting participants that the Soils and Sediments Protocol is a synthesized protocol that assembles a combination of well-established techniques. However, the data base demonstrating adequacy of components in this combination is not extensive. There is a good indication that the Protocol is applicable for preparation of both soils and sediments for mutagenicity testing. One dissenting participant expressed concern with the use of water-insoluble solvents for extraction of soils and sediments. The recovery efficiencies of methods using sodium sulfate or silica gel as drying agents and extraction with dichloromethane are not well documented and were the basis of the disagreement.

Waste Solids

There was unanimous agreement among Panel Meeting participants that the Waste Solids Protocol is a synthesized protocol that integrates a number of well-established methods and offers a number of alternative techniques. There is a good indication that these methods are applicable for preparation of soils and sediment samples for mutagenicity testing, but there is need for additional research comparing extraction techniques (i.e., Soxhlet versus blender versus sonicator), choice of solvents (use of DMSO for crude extract, single solvent, solvent series, etc.) and drying techniques (silica gel or sodium sulfate).

SECTION 2

AIR PARTICULATES

REVIEW OF THE LITERATURE

Introduction

The presence of chemicals in the air is a fact of life in a modern society, since an increasing number of chemicals have been released in large amounts into the ambient environment. The chemical industry, automobile exhaust and fossil fuel burning contribute many organics and metals to the air. Thus, the urban atmosphere contains a complex mixture of chemicals, many of which are toxic, mutagenic and carcinogenic. Also, it is not unreasonable to expect that products from reactions of these chemicals with NO_2 and SO_2, by photochemical or other processes, will be observed in the atmosphere (Calvert and Pitts 1966, Gould 1976, Hirayama et al. 1983, Leighton 1961, Lindskog 1983, Pellizzari 1978, Pitts 1983).

Many known mutagens and carcinogens have been detected in the ambient atmosphere (Broddin et al. 1980, Fishbein 1976, Singh et al. 1983, Waters et al. 1978). The atmosphere also contains many compounds whose mutagenic effect is unknown. Short-term mutagenicity test systems with a certain predictive value for carcinogenicity are now available and can be used to assess the mutagenicity of extracts of complex environmental mixtures (Harnden 1978, Waters et al. 1978). Shortly after the development by Ames (1971, 1975), of a sensitive microbiological assay for mutagenicity, Pitts et al. (1977) reported preliminary data on mutagenic activity in the organic fraction of ambient airborne particulates using the Ames assay system. In later years, many other studies were undertaken using predominantly the microbial assay system (Brusick and Young 1982, Chrisp and Fisher 1980, Drummond et al. 1982, Hughes et al. 1980, Sexton et al. 1981). Mutagenic activity was observed in ambient particulate samples from industrial or urban areas (Alfheim et al. 1983a, Commoner et al. 1978, Dehnen et al. 1977, 1981, Talcott and Wei 1977, Tokiwa et al. 1977, 1980, Hoffmann et al. 1980, Hughes et al. 1980, Krishna et al. 1983a, b, Moller and Alfheim 1980, Wang et al. 1980, Wei et al. 1980), and in particulate matter from special sources such as automobile and diesel exhausts (Huisingh et al. 1978, Kohan and Claxton 1983, Lewtas 1983, Nakagawa et al. 1983, Ohnishi et al. 1980, Rannug 1983, Rappoport et al. 1980, Schuetzle 1983, Stenberg et al. 1983a, b, Wang et al. 1978, Wei et al. 1980), fly ash from coal and other combustion processes (Alfheim et al. 1983a, b, 1984, Kubitschek and Venta 1979, Kubitschek and Williams 1980, Lofroth 1978, Moller and Alfheim 1983), coal-liquefaction products and crude and shale oil samples (Alfheim et al. 1983b, Ahlberg et al. 1983, Epler et al. 1978, Thilly et al. 1983, Vouk and Piver 1983).

A significant body of information is currently available on the methods of preparing air samples for testing with short-term mutagenicity assays (Ames testing). However, these literature procedures have not been accessible in an organized format. This review makes an attempt to integrate the available procedures into a useful and cohesive document.

Sources and Types of Air Pollutants

The chemical and biological characterization of atmospheric pollutants is of major importance in determining primary sources, elucidating chemical transformation pathways in the atmosphere and determining their potential risk to the environment and the populace. Since chemical compounds present in the atmosphere include both vapor-phase organics (VPO) (NRC 1976) and particulate organic matter (POM) (NAS 1972, NRC 1979), it is important that collection extraction and analytical techniques encompass the entire spectrum of substances (Hughes et al. 1980, Krishna et al. 1983a, b).

Air pollutants are classified as primary and secondary. Primary air pollutants are natural (dusts, vegetation, etc.) or anthropogenic in origin (smoke stack, vehicle emissions, etc.) (Fishbein 1976, Hughes et al. 1980). Secondary products may be generated from primary pollutants via atmospheric reactions (photochemical, reactions with NO_2, SO_2, O_3, etc.).

The amount of vapor-phase organics emitted anthropogenically in the United States has been estimated to be 1.9×10^{13} g/yr. The levels of vapor-phase organics are generally 10 to over 100 times greater than those of particulate organics (Duce 1978, Hughes et al. 1980).

The relative distribution of air pollutants between POM and VPO is dependent upon their vapor pressure, polarity and ambient air-temperature conditions. The successful chemical and biological analysis of the POM and VPO depends upon a number of important steps, starting with a collection and/or pre-concentration technique (Hughes et al. 1980).

Toxic and Genotoxic Chemicals Identified in Air

The insult to human health and ecosystems from airborne chemicals is due to several families of genotoxicants which include: aldehydes; aliphatic and aromatic amines; alkenes; asbestos; azaarenes (mono-, di- and polycyclic); benzene derivatives; epoxides; halogenated alkanes and alkenes; long-chain aliphatic acids, alcohols, hydrocarbons and esters; metals and their compounds; nitrosamines; NO; nitroarenes; oxidants (O_3, PAN); polycyclic aromatic hydrocarbons (PAH); SOx, etc. (Ahlberg et al. 1983, Brodzinsky and Singh 1983, Cleland 1981, Cupitt 1980, Fitzpatrick et al. 1983, Gibson 1982, 1983, Harris et al. 1982b, Henry et al. 1983, Howie and Koesters 1983, Kebbekus and Bozelli 1983, Kolber et al. 1983, Kohan and Claxton 1983, Levins 1982, Nakagawa et al. 1983, Pellizari 1978, Sawicki 1978, Singh et al. 1981, 1983, Wei and Shu 1983, West et al. 1981).

The primary sources of PAH in the United States are thought to be fuel combustion for power and heat generation and the open burning of refuse (NAS 1972). Estimates of the annual emissions of benzo(a)pyrene are up to 500 tons per year for heat and power generation and 600 tons per year for refuse burning. Of the latter figure, 140 tons per year are from forest and agricultural fires. Forest fires are also a major source of total suspended particles (TSP); estimates range from a low of 0.5 million tons per year to a high of 54 million tons per year (Ward et al. 1976).

Sources of airborne nitroarenes, which are direct-acting bacterial mutagens (in the Ames S. typhimurium assay) (1-nitropyrene, dinitropyrenes, 6-nitrobenzo(a)pyrene, etc.), include diesel-powered and gasoline-powered vehicles, woodburning fireplaces, residential heaters and coal-fired industrial furnaces (Gibson 1982, Kohan and Claxton 1983, Nakagawa et al. 1983, Pitts 1983, Zweidinger 1982). Presumably, most combustion sources emit these nitroarenes, which are thought to be formed by the reaction of NO_x gas with PAH.

Collection Techniques

The accuracy and precision of a collection and/or pre-concentration technique are important, since the materials collected must be representative of the actual composition of the vapor-phase and particulate organic matter occurring in the atmosphere (Hughes et al. 1980, Pellizzari 1978).

Collection of Particulate Organic Matter (POM)--

The collection of POM is generally done by using impactor systems, electrostatic precipitators and filtration samplers. The high-volume (Hi-Vol) sampler (which can sample approximately 2,000 m^3 of air in a 24-h period) is used routinely to collect aerosol, with nearly quantitative trapping efficiency for particles less than 20 μm in diameter (Federal Register 1971). Glass-fiber filters are commonly used for collection. A more recent method of sampling particles from very large volumes of air involves the use of the Massive Air Volume Sampler (MAVS) designed by Henry and Mitchell (1978), which samples over 2,000 m^3 of air in a 24-h period and employs impactors and an electrostatic precipitator to provide aerosol sized into three fractions (<1.7, 1.7-3.5 and >3.5 μm diameter) (Hughes et al. 1980, Kolber et al. 1983).

Electrostatic precipitation has the following inherent deficiencies: (1) The collection efficiency is dependent on a number of variables, one of which is flow rate, and sampling at the flow rate desired to collect large quantities may result in poor collection efficiency of particles, especially those of 1 μm. This is particularly significant in mutagen bioassay, since the small particles have been found to have a higher content of mutagenic material. (2) The sample is spread out over many square feet of metal collector plates, which makes quantitative retrieval difficult and artifacts due to handling more likely. (3) Particulate components may be susceptible to alteration by the electrostatic field.

An ultrahigh-volume sampler for the multiple filter collection of respirable particulate matter has been described by Fitz et al. (1983). These authors recommend the use of this ultrahigh-volume sampler when mutagen bioassay and detailed chemical analyses of the organic extract of airborne particulate matter are desirable. This ultrahigh-volume sampler is also highly recommended when short time collection intervals are desirable in order to prevent evaporation or chemical reaction of organic compounds.

Alfheim and Lindskog (1984) have recently compared different sampling systems, including electrostatic precipitation and high-volume filtration on various filters, for particle sampling. The results show that the concentrations of PAH found in the various particulate samples were in good agreement, whereas the mutagenic activity of these samples showed large variations. The highest mutagenic activity was found in the samples collected by electrostatic precipitator and on the Teflon-coated glass-fiber filters, whereas samples collected by high-volume filtration with size fractionation showed the lowest mutagenic activity. These authors do not know whether the higher activity in samples from the Teflon-coated filters, as compared to those from ordinary glass-fiber filters, represented filter artifacts or a more pronounced degradation of mutagenic compounds on the noncoated glass fiber filters.

The problem of filtration artifacts has also been addressed by Daisey et al. (1983). These authors compared fiberglass and Teflon-impregnated fiberglass filters with respect to the bacterial mutagenicity in the Ames test of extractable organic matter. Significant differences in mutagenicity between the two filter types were observed. In addition, the responses differed among bacterial strains. For example, with *S. typhimurim* TA 98, mutagenicity was greater for the polar organic fraction from the fiberglass filters; with *S. typhimurium* TA100, the polar organic fraction from the Teflon-coated fiberglass filters was greater than for the fiberglass filter fraction.

Fitz et al. (1983) have also studied the potential for the formation of artifacts during filtration sampling. Their experiments indicate that the bacterial mutagenicity as determined by the Ames bioassay did not depend significantly on the filter medium. These authors also considered the question of filter precleaning. The lack of precleaning with the extraction solvents may lead to a filter artifact which is not due to the filter type itself, but to contaminants.

Collection of Vapor-Phase Organics (VPO)--

The collection methods now available for vapor-phase organics include those techniques which trap organic vapors on sorbent surfaces (Pellizzari, 1974, 1975, 1977a, b), condense or freeze vapors in cryogenic traps (Grimsrud and Rasmussen 1975, Tyson 1975) or confine the pollutants in evacuated stainless steel bags or canisters (Hughes et al. 1980). The presence of water poses a major problem in sampling VPOs. The relative abundance of water in the atmosphere is high (as much as 100-fold compared to the organic species of interest), and the collection and analysis devices (such as gas chromatography columns, mass spectrometers, etc.) do not tolerate large quantities of water.

After careful evaluation of several sorbents for air sampling of VPOs, Pellizzari and coworkers (Bursey et al. 1977, Pellizzari 1974, 1975, 1977a, b, 1978) have recommended the following primary criteria to choose the right sorbent (Hughes et al. 1980): (1) ability to discriminate against water and preferentially concentrate the VPOs of interest, (2) minimal decomposition or polymerization of the sample constituents during collection and recovery, (3) high breakthrough volumes, (4) quantitative collection efficiency and recovery of trapped vapors and (5) collection systems that do not contribute to *in situ* formation of artifacts.

Activated carbon or charcoal has been used as a sorbent material in some VPO studies (Eppig et al. 1982, Grob and Grob 1971). However, the recovery of the collected VPOs from charcoal is usually incomplete, and in some cases, charcoal catalyzed reactions between individual organics present in the complex mixture (Miuere and Dietrich 1973, White et al. 1970). In the cryogenic (freeze-out) collection technique (generally employed for C_2-C_8 hydrocarbons), the very volatile VPOs are generally recovered from the frozen sample by distillation. The presence of water continues to pose technical limitations, and the VPOs in the cryogenic trap are usually separated from water vapor through a sequential increase in temperature before analysis.

Recent literature highly recommends the use of the Tenax-GC adsorbent (a porous polymer of 2,6-diphenyl-p-phenylene oxide) collection technique for the C_6-C_{15} volatility range of VPOs (Adams et al. 1977, Bursey et al. 1977, Harris et al. 1982b, Kaiser 1974, Lutz 1982, Pellizzari 1974, 1975, 1977a, b, 1978, Walling et al. 1982).

A recent report by Pellizzari et al. (1983) describes the preparation of 61 different polyimide sorbents that have the potential of either replacing or complementing Tenax-GC. According to this report, four polyimide sorbents, PI-109, PI-115, PI-119 and PI-149, are selected for further studies on collection desorption efficiencies, artifact formation, shelf life, humidity and the effects of inorganic species such as ozone, nitrogen oxides and sulfur oxides.

The use of several other resins such as XAD-2, XAD-7, chromosorb 100 series, Porapak, Florisil, Ambersorb-340, Ambersorb-347 and Ambersorb-348 has also been recommended for the sampling of VPOs in air (Harris et al. 1982a, Lutz 1982). Florisil is comparable to XAD-2 and Tenax-GC in terms of its volumetric capacity for collection of the alkanes, alcohols and chlorinated aromatics. Florisil shows very much higher (about 10,000 times) capacity for collection of volatile chlorinated aliphatic compounds such as dichloroethanes and dichloropropanes. Ambersorb XE-340, Ambersorb XE-347 and Amberlite XAD-7 have a substantially greater capacity for collection of low molecular weight alcohols than do XAD-2 and Tenax-GC.

Preparation of Extracts for Bioassays

Introduction--

As Guerin et al. (1978) point out, a major consideration in the mutagenicity testing of complex chemical mixtures is that such mixtures contain chemicals of a wide variety of classes and an equally wide range of concentrations that are potentially capable of causing additive, antagonistic or synergistic responses in test organisms or at specific receptor sites. Such interactions may be a function of the combined dose of toxicants or of the inherent genetic susceptibility of a particular target organism. The preparation of complex mixtures for *in vitro* bioassay (e.g., Ames test) is complicated by at least two factors: (1) the relevance of the material applied to the test system and (2) the compatibility of the material to the test system (Guerin et al. 1978). Chemical "relevance" is achieved when the test system is dosed with a material whose chemical composition mimics that which reaches the natural point of impact. Difficulties with "compatibility" are often encountered when the material being bioassayed contains constituents which interfere with the test organisms' ability to respond to the effect of interest. High concentrations of mildly toxic constituents or small quantities of highly toxic constituents can sometimes mask the more subtle effect of mutagenic constituents. Any steps taken to remove toxic constituents in order to make the material compatible with the test system necessarily involve a change in the physical or chemical nature of the test material. Thus, the objective of selecting the best sample preparation protocol is to choose a procedure which prepares materials in a form suitable for biotesting with a minimal or at least interpretable impact on the relevance of the test material.

Solvent Extraction of POM Samples--

Sample preparation is a costly feature in mutagenicity testing. When chemical purification is not the goal, samples of POM can be extracted with organic solvents and the crude extracts tested for mutagenicity without chemical fractionation. Fractionation is only required if the crude extract proves to be too toxic for direct testing. Thus, a stepwise approach to sample prefractionation is the most efficient.

Direct Extraction--

Direct extraction of air particulates without fractionation is highly recommended. In a recent study by Krishna et al. (1983a), organic materials were extracted from airborne particles by shaking with different solvent systems (without fractionation), including acetone, benzene, cyclohexane, methylene dichloride, methanol, a mixture of acetone and methylene dichloride and a combination of benzene, cyclohexane and methanol. The solvent-extracted materials were tested for mutagenic activity with the Ames Salmonella/microsomal assay system. Acetone- and cyclohexane-extracted materials gave the highest and lowest mutagenic activities, respectively. In addition, these authors tried sequential extraction with acetone followed by methylene dichloride, which gave a better mutagenic response than acetone alone or acetone in combination with methylene dichloride.

Even though acetone has been used widely in different laboratories for air particulate extraction, it is not a highly recommended solvent due to its reactivity with other chemicals and unavailability in very pure form (Lentzen et al. 1978, Brusick and Young 1981).

Other solvent systems, such as cyclohexane (Alfheim and Moller 1979), benzene-hexane-isopropanol mixture (Commoner et al. 1978), cyclohexane, methylene chloride and acetone serially (Daisey et al. 1979), methanol (Dehnen et al. 1977), methanol-benzene-methylene chloride mixture (Pitts et al. 1977, Preidecker 1980), acetone (Talcott and Wei 1977) and benzene (Fukino et al. 1982, Teranishi et al. 1978), have also been used for the extraction of mutagenic material from ambient air particulates. Among these solvents, dichloromethane is the best choice for air particulate extraction, due to its intermediate polarity.

Among such different modes of extraction as shaking, Soxhlet extraction and sonication, either sonication or Soxhlet extraction seems to be the mode of choice for the extraction of mutagens from airborne particulates (Hughes et al. 1980, Krishna et al. 1983b, Alfheim and Lindskog 1984).

Separation and Identification of Chemicals for Biological Testing

Separation--

Chemical fractionation--As mentioned earlier, fractionation is required if the crude extract proves to be too toxic for direct mutagenicity testing. Fractionation is also needed for chemical purification and identification by chromatographic (gas and liquid) techniques and mass spectrometry.

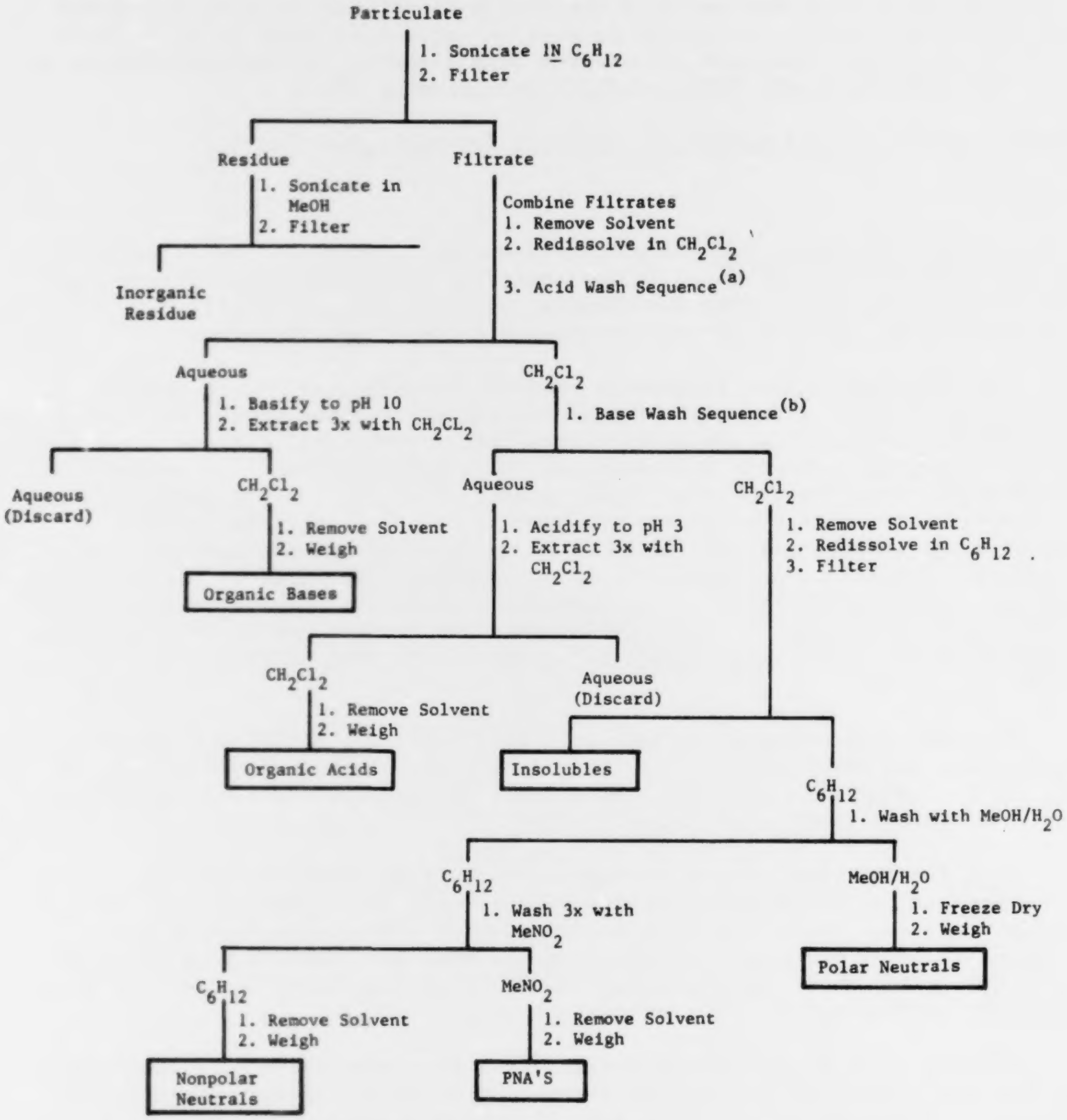
The partition scheme (Figure 1) proposed by Pellizzari et al. (1978) is one of the methods of fractionation of ambient air particulates. This scheme results in fractionating air particulates into six chemical classes: acids, bases, polycyclic aromatic hydrocarbons, polar neutrals, nonpolar neutrals and insolubles. This fractionation scheme essentially involves the sonication of air particulates with cyclohexane to separate solids (containing polar organics and inorganics) and the cyclohexane layer containing nonpolar organics. The solids and the cyclohexane layer undergo further fractionation through a solvent partition scheme (methanol, methylene dichloride and aqueous acids for solids; aqueous acids, cyclohexane and aqueous methanol for nonpolar organics in the cyclohexane layer) to produce six fractions (Pellizzari et al. 1978).

This partition scheme has been validated for its chemical efficacy by using chemicals representative of individual chemical classes (Pellizzari et al. 1978). Using this fractionation scheme, the various chemicals used were recovered with a high percentage efficiency.

As a further check on the procedure, thin-layer chromatography (TLC) scans were conducted on each fraction to ascertain the extent, if any, of spillover of one compound into other fraction(s). No such spillover was detected by these authors. The first sample that was examined very successfully using this fractionation scheme was collected at South Charleston, West Virginia, during August, 1977 (Pellizzari et al. 1978).

Several other fractionation schemes have also been developed, including (1) isolation and fractionation of organics from diesel exhaust particulates (Figure 2) (Huisingh et al. 1978) and (2) acid-base extractive separation of complex shale oil fractions (Figure 3) (Guerin et al. 1978).

Separation by chromatographic techniques--
Column chromatography: This is the most widely used separation method for POM. A large variety of adsorbents, such as alumina, silica gel, Florisil, cellulose acetate and gel materials, have been used for separation. Adsorbents with uniform particle size, 60-80, 80-100 and 100-120 mesh, and


Particulate
1. Sonicate 1N C6H12
2. Filter
Residue
Filtrate
1. Sonicate in MeOH
2. Filter
Inorganic Residue
Combine Filtrates
1. Remove Solvent
2. Redissolve in CH2Cl2
3. Acid Wash Sequence(a)
Aqueous
CH2Cl2
1. Basify to pH 10
2. Extract 3x with CH2CL2
1. Base Wash Sequence(b)
Aqueous (Discard)
CH2Cl2
Aqueous
CH2Cl2
1. Remove Solvent
2. Weigh
Organic Bases
1. Acidify to pH 3
2. Extract 3x with CH2Cl2
1. Remove Solvent
2. Redissolve in C6H12
3. Filter
CH2Cl2
Aqueous (Discard)
1. Remove Solvent
2. Weigh
Organic Acids
Insolubles
C6H12
1. Wash with MeOH/H2O
C6H12
MeOH/H2O
1. Wash 3x with MeNO2
1. Freeze Dry
2. Weigh
Polar Neutrals
C6H12
MeNO2
1. Remove Solvent
2. Weigh
1. Remove Solvent
2. Weigh
Nonpolar Neutrals
PNA'S

(a) Acid wash sequence 2x with 10% H_2SO_4, 1x with 20% H_2SO_4.

(b) Base wash sequence 3x with 1N NaOH.

Figure 1. Fractionation scheme for the extraction of chemicals from ambient air particulates (adapted from Hughes et al. 1980).

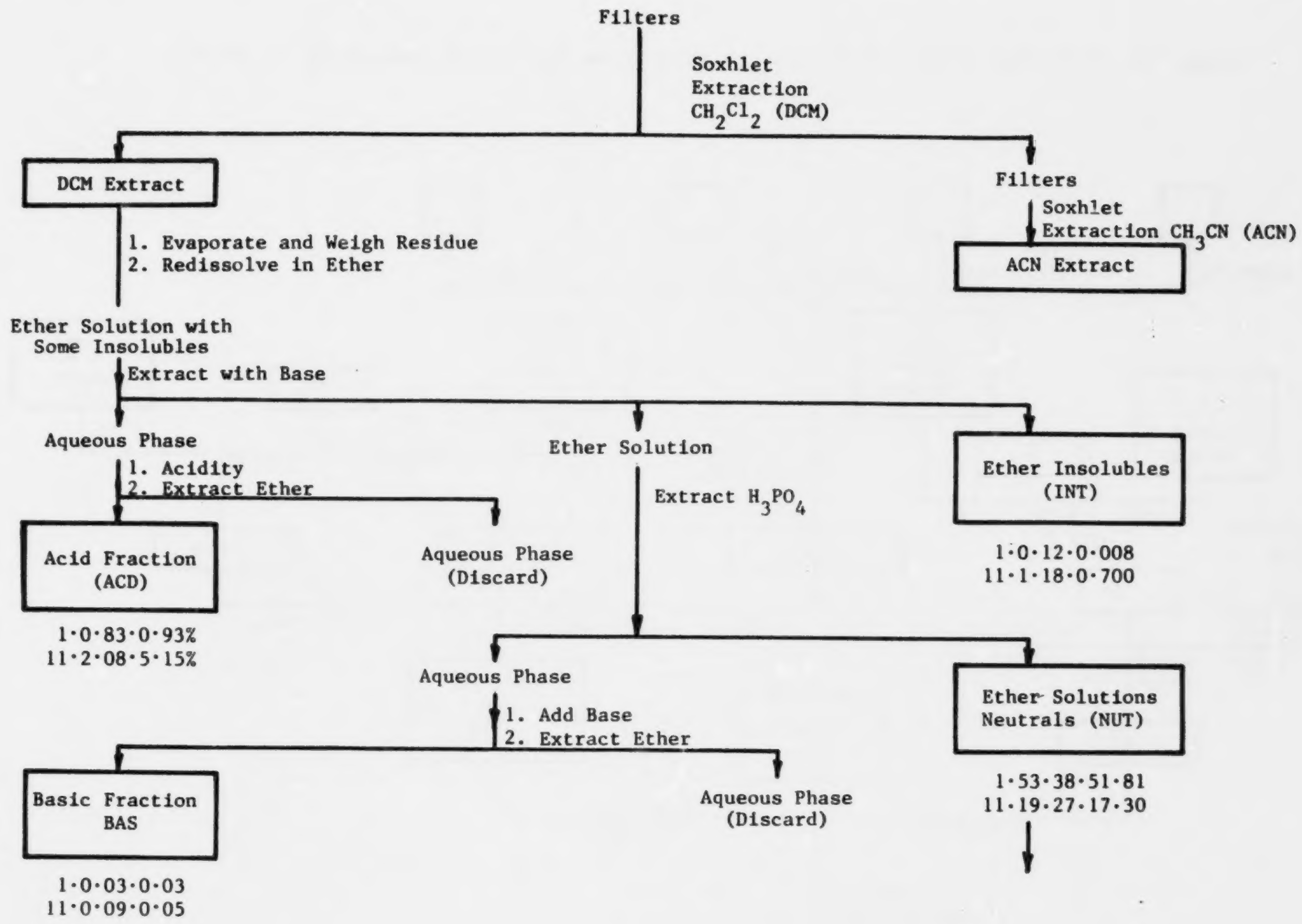

Filters
Soxhlet
Extraction
CH_2Cl_2 (DCM)
DCM Extract
Filters
Soxhlet
Extraction CH_3CN (ACN)
ACN Extract
1. Evaporate and Weigh Residue
2. Redissolve in Ether
Ether Solution with
Some Insolubles
Extract with Base
Aqueous Phase
Ether Solution
Ether Insolubles
(INT)
1. Acidity
2. Extract Ether
Extract H_3PO_4
Acid Fraction
(ACD)
Aqueous Phase
(Discard)
1·0·12·0·008
11·1·18·0·700
1·0·83·0·93%
11·2·08·5·15%
Aqueous Phase
Ether Solutions
Neutrals (NUT)
1. Add Base
2. Extract Ether
Basic Fraction
BAS
Aqueous Phase
(Discard)
1·53·38·51·81
11·19·27·17·30
1·0·03·0·03
11·0·09·0·05

Figure 2. Isolation and fractionation of organics from diesel exhaust particulates (Huisingh et al. 1978).

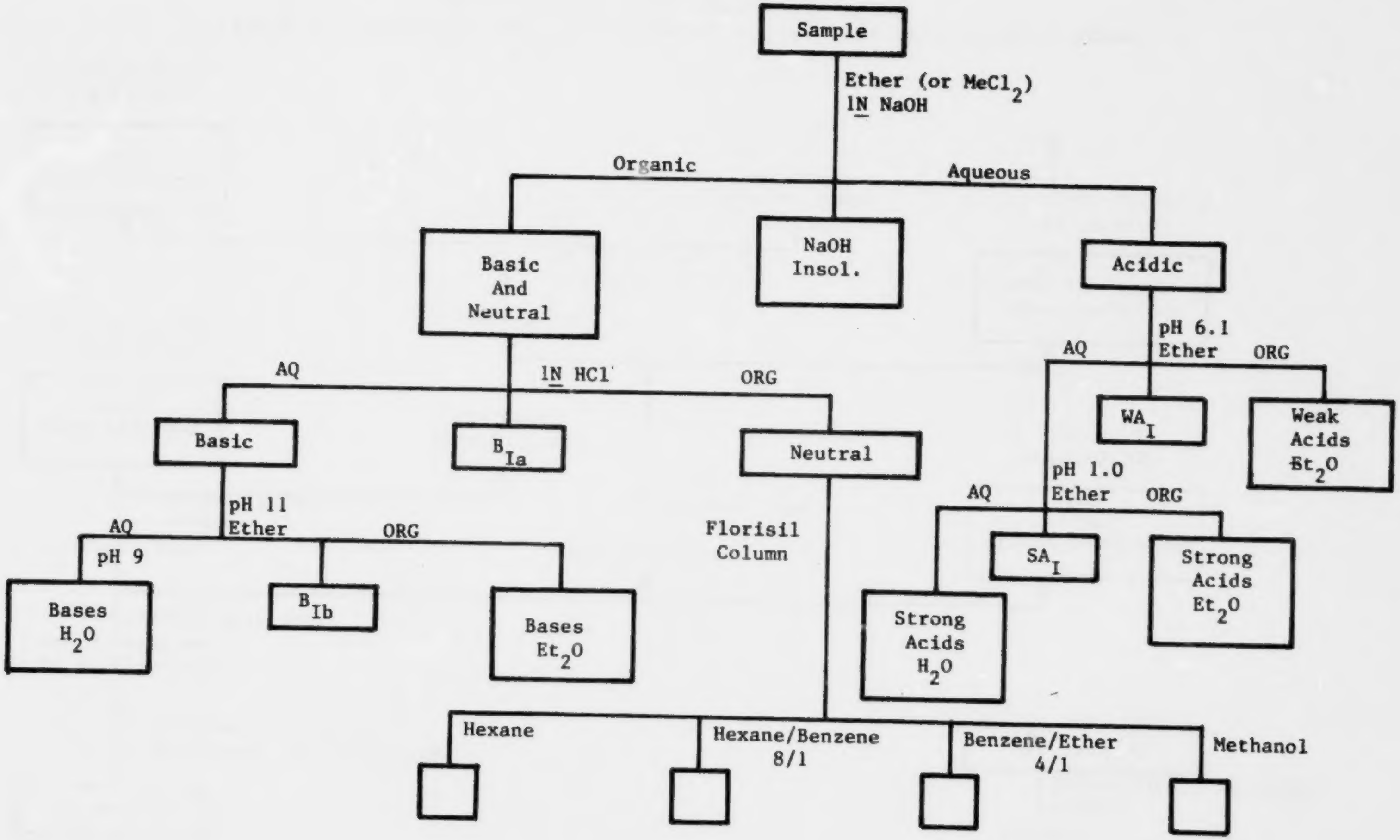

Sample
Ether (or $MeCl_2$)
1N NaOH
Organic
Aqueous
Basic And Neutral
NaOH Insol.
Acidic
AQ
1N HCl
ORG
Basic
B_{Ia}
Neutral
pH 11 Ether
AQ
pH 9
ORG
Bases H_2O
B_{Ib}
Bases Et_2O
Florisil Column
Hexane
Hexane/Benzene 8/1
Benzene/Ether 4/1
Methanol
pH 6.1 Ether
AQ
ORG
WA_I
Weak Acids Et_2O
pH 1.0 Ether
AQ
ORG
SA_I
Strong Acids Et_2O
Strong Acids H_2O

Figure 3. Acid-base extractive separation of complex mixtures (Guerin et al. 1978).

column diameter:length ratios of at least 1.25 are suggested. The laboratory temperature should be kept reasonably constant, and the solvent used for elution should be of highest purity. Maximal separation can be achieved by slowly and evenly increasing the polarity of the eluting solvents. The hydrocarbons are eluted from the column in the following order: Aliphatics, olefins, benzene derivatives, naphthalene derivatives, dibenzofuran fraction, anthracene fraction, chrysene fraction, benzopyrene fraction and coronene fraction (Sawicki et al. 1970).

Thin-layer chromatography: This technique is quick, inexpensive and reasonably reproducible and is one of the better methods for the separation of isomeric compounds. With the availability of in situ scanning techniques (thin-layer scanner), this method has been used for quantification of POM with reasonably good accuracy. A number of materials have been used as sorbent materials for the plates, including silica (Liberti et al. 1975, Candeli et al. 1975, Hunter 1975, Dong et al. 1976); alumina (Woidich et al. 1977, Sawicki et al. 1970a, b); cellulose (Sawicki et al. 1967); cellulose acetate (Schultz et al. 1973, Tomingas et al. 1977, Harrison and Powell 1975, Woidich et al. 1977) and polyamide (Bories 1977). A number of modifications, including silanization of silica gel (Brocco et al. 1973) and channel thin-layer chromatography (Zoccolillo and Liberti 1976), have been proposed. In the latter procedure, the components which need to be fractionated on a thin-layer plate are made to flow into narrow development channels in order to prevent the spreading of the chromatographic spots.

Both PAH and polynuclear aza-heterocyclic compounds have been separated by the TLC method. A large number of variations of the TLC procedure, such as variation of developing solvents, use of a mixture of sorbents and development in one or two dimensions, have been used to affect better resolution of the components. The effect of solvent variations on cellulose acetate TLC has been discussed in detail by Woidich et al. (1977). Although alumina-cellulose (2:1) has been used as a composite adsorbent, the best resolution is obtained when two-dimensional TLC on a composite plate of aluminum oxide-40% acetylated cellulose (2:1) is used (Chatot et al. 1972a,b, Pierce and Katz 1975).

A large number of aza-heterocyclic compounds (acridines and quinolines) found in urban airborne particulates and in air pollution source effluents have been separated by TLC procedure. The basic fraction from a coal-tar-pitch sample was subjected to a two-dimensional TLC separation on silica gel-cellulose (2:1) with pentane-ether (9:1, v/v) and dimethyl formamide-water (35:15, v/v) solvent systems (Sawicki et al. 1965).

Two-dimensional TLC separation has also been successfully used during the quantification of acridine, benz(c)acridine, 7Hbenz(de)anthracen-7-one and phenalen-1-one in airborne particulate samples (Sawicki et al. 1966).

There are several disadvantages with the TLC procedure. Mass spectrometry and liquid chromatography, when applied as additional analytical tools for identification of POM, have demonstrated that the individual spots or bands are sometimes mixtures of two or more compounds. Also, the TLC procedure cannot be used when separation of a large number of compounds is required since TLC resolution is limited. Furthermore, during the TLC procedure, trace amounts of compounds may photodecompose, requiring addition of an internal standard to correct for the losses.

Gas chromatography: The closeness of the chemical structure among some of the POM and their relatively high boiling points demand the stringent requirements of high resolution and thermal stability at high temperature for the chromatography column packings. Unfortunately, until recently, the available liquid phases that were thermally the most stable were barely adequate for the temperature required to separate some of the high-boiling PAHs. However, a large variety of column packing materials has since been used for POM separation. Depending on their mode of operation, the gas chromatographic methods used so far can be divided into: (1) packed column and (2) capillary column, e.g., surface-coated open tubular column and wall-coated open tubular column.

Packed column: A number of packing materials have been used in packed column chromatography. A gas-solid chromatographic phase using Chromosorb (Mitera et al. 1977) and GLC (gas-liquid) phases using OV-1 (Burchfield et al. 1974, Lao et al. 1975), OV-7 (Sawicki et al. 1966), OV-25 (Griest et al. 1975), polymetaphenoxylene (Harrison and Powell 1975), apiezon L (Sawicki et al. 1966, DeMaoi and Corn 1966), apiezon W (Griest et al. 1975), SE-30 (Sawicki et al. 1974, DeMaio and Corn 1966, Shushunovo et al. 1975), SE-52 (Chakraborty and Long 1967, Chatot et al. 1972), Dexsil-300 GC (Cautreels and Van Cauwenberghe 1976, Burchfield et al. 1974), Dexsil-400 GC (Lao et al. 1975), Dexsil-410 GC (Lao et al. 1975) and nematic liquid crystals (Strup et al. 1976, Janini et al. 1975, 1976) have been used as packing materials.

One of the superior column packing materials available for POM separation is Dexsil-300 or Dexsil-400. The column bleeding characteristic and resolution capability for these packing materials make them highly suitable for POM analysis. The resolution of Dexsil-400 is even better than that of Dexsil-300 (Lao et al. 1975). The separation of both PAH and aza-arenes in samples containing coal tar has been accomplished with these pecking materials (Lao et al. 1975). Dong et al. (1977a,b) have used a Dexsil-300 column for the separation and quantitation of a number of aza-arenes in the basic fraction of airborne particulate matter.

Capillary column: These columns are more advantageous than packed columns due to their ability to affect higher resolution. The number of theoretical plates obtained from such columns may vary between 20,000-70,000 (Cantuti et al. 1965). Versamid-900 (Brocco et al. 1973), SE-30 (Cantuti et al. 1965), XE-60 (Cantuti et al. 1965), SE-52 (Bartle et al. 1974, Colucci and Begeman 1971, Novotny et al. 1974, Hites 1975, Bartle et al. 1976), SE-54 (Bjorseth 1978), Dexsil-300 (Jones et al. 1976), Emulphor ON 870 (Bertsch et al. 1974), carbowax-20 polymer (Hill et al. 1977), OV-1 (Lunde and Bjorseth 1977), OV-17 (Grimmer et al. 1972), OV-101 (Grimmer et al. 1972, Harke et al. 1976, Panalaks 1976) and apiezon L (Liberti et al. 1975, Candeli et al. 1975) have all been used as packing materials for capillary columns. The most widely used column for separation of POM is SE-52. Cantuti et al. (1965)

compared several liquid phases and found that a 50-m SE-52 column was most effective for POM separation and had a theoretical plate value of 40,000. Investigations by Bjorseth (1978) have demonstrated that capillary columns using SE-54 as the stationary phase have excellent separation efficiency, low column bleed and long-term stability. Both PAH and aza-arenes have been separated with this packing material (Bjorseth 1978). A 40-m capillary column with Versamid-900 as the stationary phase is very effective for the separation of aza-arenes (theoretical plate value of 60,000) (Brocco et al. 1973).

High performance liquid chromatography (HPLC): The ability of HPLC to separate POM compounds not normally accomplished by gas chromatography (GC) indicates the great potential of this method for POM analysis. In addition, HPLC offers many advantages over GLC: it operates at much lower temperatures, fraction collection is simple and convenient, the capacity to accept sample is high and fluorescence spectroscopic detection demonstrates high sensitivity and selectivity for POM compounds.

In HPLC, the mobile phase modifier is the most powerful variable for changing retention times. The choice of mobile phase is dictated by the mode of separation accomplished by the sorbent. In the normal mode, hydrocarbon solvents are generally used as the mobile phase. Any solvent that dissolves POM in fair amounts, is miscible with water and is transparent at UV absorption wavelengths (for UV or fluorescence detector) can be used as a modifier in the reverse phase-HPLC.

It should be mentioned that solvent programming has proven to be a powerful tool in separating the compounds from each other. Several researchers have taken advantage of increased temperature (70 to 80 C) of the column to obtain a similar objective. At higher temperature, reduced viscosity of the mobile phase increases column efficiency and decreases relative retention times. The sample capacity is also increased at higher temperatures, allowing larger amounts of sample injection. The most versatile columns for the analysis of PAHs in POM operate on the principle of reversed-phase mechanism. The C-18 reversed-phase column has been applied for the separation of both PAH (Dong 1976, Flessel et al. 1981, 1984) and aza-arenes (Dong et al. 1977). Normal-phase HPLC columns have also been used for separating extracts from diesel exhaust particles (Schuetzle et al. 1981, 1982), woodstove samples (Alfheim et al. 1984) and ambient air particles (Eisenberg 1978; Ramdahl et al. 1982).

HPLC offers several advantages, including higher resolution and lower operating temperature than gas chromatography. The method is nondestructive, and the injected sample can be recovered easily. This technique is gaining wide acceptance as a modern method for the separation of organics in POM, although the separation of isomeric PAHs from real-life samples may not be complete. Further research is needed in this area. Caution must be exercised before emphasizing the HPLC method for the fractionation of complex environmental samples in the place of other conventional fractionation techniques discussed earlier.

Mutagenicity of Vapor-phase Air Pollutants

No data exist on the mutagenicity of vapor-phase air pollutant complex mixtures collected on-site and tested in the laboratory. However, some studies have investigated the mutagenicity of pure compounds, such as vinyl chloride (Loprieno et al. 1976, Malaveille et al. 1975, 1976), SO_2 (Ma et al. 1973) and ozone (Zelac et al. 1971), which are often encountered as components of vapor-phase air pollutants.

The Ames *Salmonella*/microsome assay is not adequate to detect mutagenicity of compounds with high vapor pressure because this technique requires incubation of the sample in agar at 45 C. Chemicals which boil below this temperature will volatilize before the system has an opportunity to form mutagenic compounds. Rannug et al. (1976) have utilized preincubation of the bacteria and S9 (1 hour at 25 C) to study the metabolites of vinyl chloride. Rosenkrantz et al. (1978) have used a modified spot test to detect vapors: discs impregnated with volatile sample were incubated as usual, or the discs were placed in sealed plastic bags and incubated. The limitation of this procedure include: nondiffusible test agents will not be detected and quantitation is difficult. There is no currently available way of testing VPOs in the standard Ames *Salmonella* assay. Thus, no protocol is currently available for VPOs.

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AIR PARTICULATES WORKGROUP REPORT

Workgroup Tasks

Summary of Tasks--

Consider question of Vapor Phase and/or Particulate Organics.

Review and evaluate sampling techniques for air particles. How much sample is needed?

How to prepare crude extracts for bioassay?

How to further fractionate crude extracts (to separate toxic from mutagenic components).

Key Peer Review Comments--

(addressed by protocol)

Summary of Workgroup Progress--

Mutagenicity protocols for VPOs - not available.

Air particle collection techniques are source dependent; thus, a general collection technique cannot be specified in detail here. However, a specific protocol for Hi-Vol sampling of ambient air particulate is described. A scheme for sample preparation protocol is enclosed (Figure 4).

A general scheme for the preparation of crude organic extracts from air particles can be recommended.

Schemes for chemical fractionation of crude air particulate extracts are source-dependent; thus, specific fractionation protocol cannot be recommended (at this time). Further research in LC/HPLC methodology is recommended.

Summary of Key Workgroup Discussions

Major Consensus Opinions--

Air protocol to focus on particles; VPOs will not be considered.

Air particle collection techniques are sufficiently source-dependent to preclude development of a generalized particle collection protocol. However, for application to ambient air particle monitoring, the use of standard Hi-Vol sampling is recommended; i.e., community air particulate from 24-h Hi-Vol sampler will provide sufficient sample for minimum characterization on genotoxicity in standard Ames test, but not enough for detailed chemical analyses.

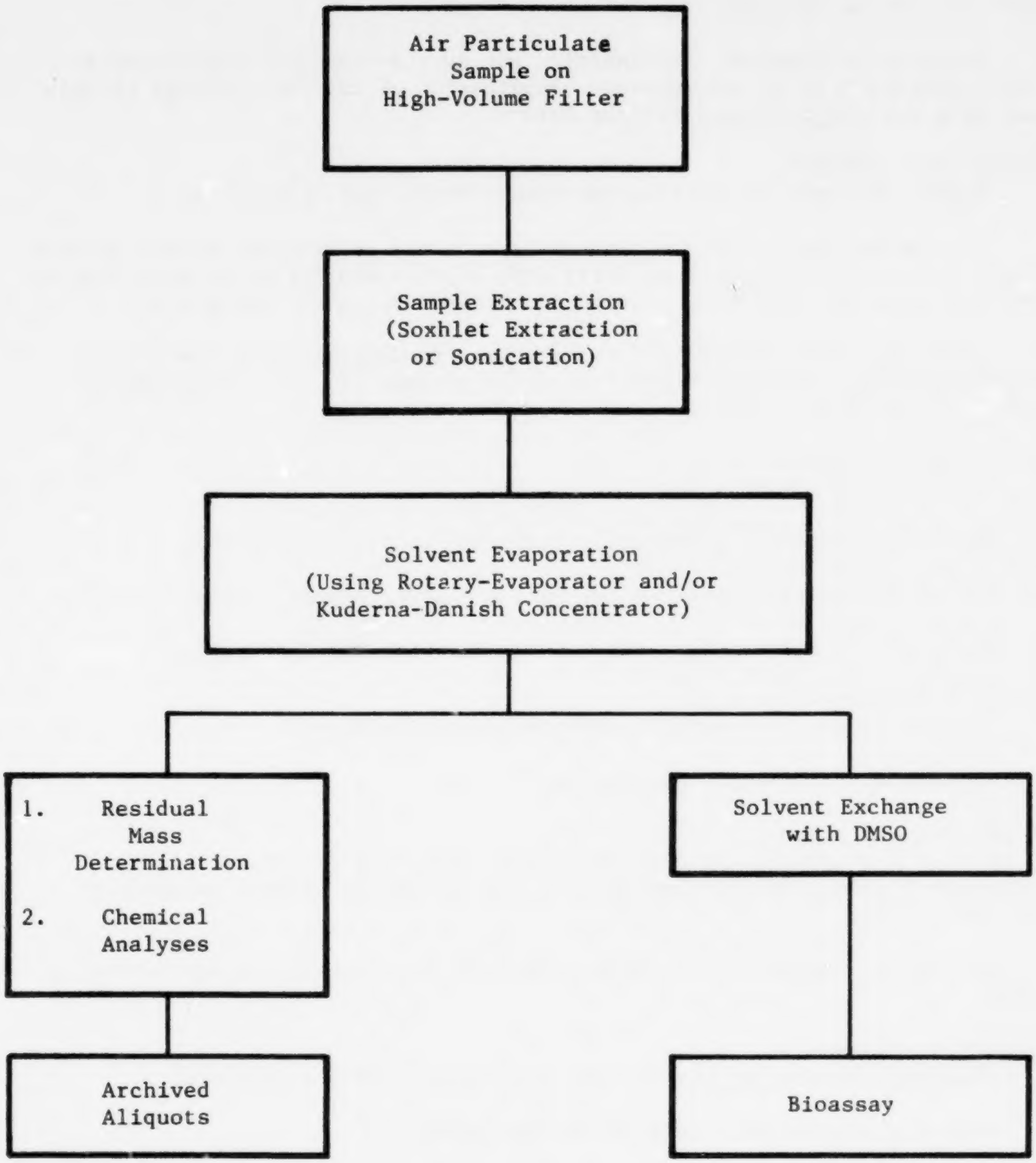

Air Particulate Sample on High-Volume Filter
Sample Extraction (Soxhlet Extraction or Sonication)
Solvent Evaporation (Using Rotary-Evaporator and/or Kuderna-Danish Concentrator)
1. Residual Mass Determination
2. Chemical Analyses
Solvent Exchange with DMSO
Archived Aliquots
Bioassay

Figure 4. Flow scheme for the preparation of samples from ambient air particulates.

A general protocol for preparation of crude organic extracts of air particles can be proposed (see below).

Schemes for chemical fractionation are source- and goal-dependent; no single protocol can be recommended. Application of HPLC methodology to this task is a key research need for the future.

Unresolvable Issues--

Issue: Minimum Air Particulate Sample Size--"Scientific" Basis

If bioassay only, then sufficient particulate on standard Hi-Vol in 24-h (every 12-h), so feasible to do daily with diurnal monitoring studies (but no chemical analysis, including gravimetric determination and archiving).

If bioassay and chemistry (with gravimetric determination) required, then must either composite Hi-Vols or scale up sampler, e.g., Ultra Hi-Vol sampler (Fitz et al. 1983).

Minimum Sample Size--

Workgroup Recommendation:

Use 24-h Hi-Vol for genotoxicity testing--routine monitoring.

Hi-Vol compositing required for full bioassay/chemical characterization.

Use Ultra Hi-Vol sampler, but not feasible for routine applications.

Choice of Solvent--

Single solvent, sequential extraction, mixed solvent.

Recommend side-by-side evaluation.

Solvent Removal Steps--

Use of K-D and solvent exchange steps: Are they necessary? Each introduces higher level of complexity - good to obtain minimum acceptable protocol.

Recommend comparison with/without K-D and with/without solvent exchange steps.

Fractionation Scheme--

Acid-Base-Neutral or LC--methods to be developed for toxicity?

Emphasis of LC methodology--to be developed.

Evaluation of Proposed Protocol

Adequacy--

Adequate for preparation of crude extracts. Inadequate for fractionation.

Validity--

To be validated.

Limitations--

Some steps in protocol require validation.

Comparison to Other Five Media Protocols--

Common problem of fractionation scheme.

Other Data or Information Requirements

Information Gaps--

Sample collection: Hi-Vol "A," Ultra Hi-Vol "B."

Preparation of crude extracts. "B" - QC data needed.

1. Solvent extraction comparisons.
2. Solvent removal issues.

Fractionation of crude extracts. "C" - Research needed.

Research Program Needs--

Rationale for need - fractionation scheme necessary for toxic samples and chemical characterization.

Alternatives and variables - not available.

PROTOCOL FOR PREPARATION OF AIR PARTICULATES FOR MUTAGENICITY TESTING

1.0 Scope and Application

1.1 Introduction--

Implementation of this approach must include a recognition that it is impossible to accommodate every conceivable condition encountered in preparing air or source samples for Ames biotesting. Procedures for the preparation of particulate samples are developed to the point where a unified approach can be recommended. Techniques for the preparation and testing of vapor-phase organics (VPOs) and semivolatile organic materials are still in a research phase (see the Review) and will not be covered by the protocol proposed here.

The procedures described are intended to be used under the direction of a professional experienced in the sampling, preparation and analysis of complex environmental analyses.

1.2 Scope and Application--

1.2.1 The methods described here can be used for the isolation/preparation of residue organics from air particulates. This protocol has the following applications:

a. Routine air quality characterization to determine the environmental quality (by EPA, Army, etc.).

b. Routine monitoring of community air and observation of seasonal trends in order to get data for correlating the geographic distribution of cancer incidence with air pollutants.

1.2.2 The methods proposed in this protocol are limited to direct application to high-volume ambient air particulate samples. Detailed procedures are provided for this application. Indirect application, with modifications, is possible to most other ambient or source particulate samples. Modifications to this procedure are not included, since the types of samples and associated modifications are source-specific and, while similar to the detailed procedure, will vary with the source of the air particulates and the type of sampling technique employed.

2.0 Summary of Method

2.1 Air particles (1 g) are extracted twice with dichloromethane (100 mL) by either Soxhlet extraction (5-10 cycles/hour for 16 hours at 40 C) or sonication (at room temperature for 15 minutes each time). The dichloromethane extract is filtered through a 0.5-μm Fluoropore filter (Millipore Corporation) into a round-bottom evaporating flask and concentrated to 10 mL in either a rotary evaporator or a Kuderna-Danish concentrator. One portion of this extract (5 mL) is saved for mass determination by gravimetric analysis and chemical analyses. The other portion of the extract (5 mL) is solvent exchanged with dimethyl sulfoxide (5 mL) under a gentle stream of nitrogen, and the final volume is noted. This DMSO solution is further diluted to appropriate concentration and bioassayed.

2.2 It is recommended that the air samples be stored in the dark at -20 C, and the solvent (dichloromethane or DMSO) extracts in amber-colored Teflon screw-capped bottles at -20 C. These air samples and solvent extracts should be thawed to room temperature prior to use.

3.0 Definitions

3.1 Air Particulates--

Air particulates are defined operationally as air particles having 20-μm aerodynamic diameters.

Recent studies (Talcott and Harger 1979, Preidecker 1980) have shown that most of the mutagenic activity associated with air particulate samples is found in particles of $\leq$2-μm aerodynamic diameter. This is important when one considers that the penetration of particles into the lung is inversely related to particle size, i.e., most of the larger particles are removed in the upper part of the respiratory tree (Hatch and Gross 1964). According to Pierce and Katz (1975), approximately 70% to 90% of the total polynuclear aromatic hydrocarbon (PAH) content of airborne particles is associated with particles of $\leq$5-μm diameter.

4.0 Interferences

4.1 Sampling Artifacts--

4.1.1 Non-incorporation of sampling efficiency-- The evaluation of sampling efficiency is particularly difficult under ambient atmospheric sampling conditions. The percent recovery of a collected sample, however, is rarely determined during either ambient or source sampling. The lack of incorporation of sampling efficiency lends considerable uncertainty in the reported POM levels.

4.1.2 Evaporative losses and reactive conversion--Collection of POM on widely used glass-fiber filter alone without the backup adsorbent is subject to two kinds of errors. First, lower molecular weight compounds on adsorbed particulate phase may have some equilibrium vapor concentrations under atmospheric conditions. This vapor part will escape collection on the filter. Second, air passing through a filter containing the collected substances will carry away amounts equal to or less than the equilibrium vapor concentration because of desorption from the adsorbed substrate. Pupp et al. (1974) determined the equilibrium vapor concentration for a number of polycyclic aromatic hydrocarbons, and concluded that considerable losses occur during ambient sampling of compounds having an equilibrium vapor concentration of 0.5 x 10 mg/m or higher at ambient temperature. Considerable losses are expected with pyrene, anthracene, phenanthrene and benzo(a)anthracene.

Besides sublimation, chemical changes of POM can occur on the sampling probe and on the filter. The first of these chemical changes may occur as the result of catalytic effects of the probe material used during sampling. Bermann (1974) studied this effect and concluded that neither Vycor nor stainless steel used as probe material causes any catalytic effect in promoting chemical reactions. However, there are many oxidants (such as NO_2, O_3, etc.) in the air which are drawn through the filter during sampling. Using simulated atmospheric conditions, Lane and Katz (1977) have demonstrated that polycyclic aromatic hydrocarbons coated on a glass surface will be rapidly oxidized by the prevailing oxidants in the atmosphere. Their work also indicates that benzo(a)pyrene is more prone to oxidation than benzo(b)fluoranthene.

Pitts and co-workers (Pitts et al. 1978) have shown that certain polycyclic aromatic hydrocarbons coated on glass fiber filters are transformed into mutagenic nitroderivatives by exposure of aromatic compounds to nitrogen dioxide.

4.2 Losses During Transportation and Storage--

Another source of error that needs to be properly addressed is the loss of POM during transport and storage of the collected samples. Losses during these periods can occur from three sources: (1) photooxidation, (2) volatilization and (3) chemical oxidation. The first of these losses can be easily prevented by transporting and storing the sample in the dark. Losses of POM due to the other two factors have been noted by various investigators. A 40% to 60% decrease in the concentration of fluoranthene, phenanthrene and pyrene and the rearrangement of dibenzo(a,l)pyrene to dibenzo-(a,e)fluoranthene and benzo(a)anthracene to chrysene have been noted when the sample is stored for three weeks (Butler, 1975). Hermann (1974) has noted considerable error due to degradation or reaction when the sample is stored at room temperature and exposed to air. For these reasons, the POM samples should be stored in the dark and at low temperature (-20 C) as particles, rather than as the organic extract, until solvent extraction.

4.3 Sample Preparation--

Several errors can be introduced during sample preparation stage.

4.3.1 Solvent purity and reactivity--The use of impure solvents for extraction can cause positive or negative interferences. Solvents of very high purity (spectroscopic or pesticide grade) should be used for extraction of POM from air particulates. One of the reasons for not recommending acetone for extraction is that it cannot be purchased in very high purity form. In addition, acetone is known to be reactive and can cause structural modifications during extraction.

4.3.2 Incomplete evaporation of solvent--Incomplete evaporation of the highly recommended dichloromethane solvent may tend to produce toxicity or increase the mutagenic activity of the extract since dichloromethane is a mutagen. Hence, the complete removal of the solvent is highly recommended.

4.4 Other Sources of Error--

The use of all-glass (Pyrex) apparatus and glassware will minimize error due to contaminations.

Appropriate solvent blanks have to be run simultaneously in order to see whether any artifact has been introduced in the mutagenicity determination.

5.0 Safety

5.1 *In vivo* bioassays have demonstrated that the greatest carcinogenic activity of organic pollutants in air is associated with the fraction containing both polynuclear aromatic compounds and their nitrogen analogs, the nitro- and aza-arenes. Since the toxicity or carcinogenicity of chemicals used or extracted in this method has not been precisely defined, each chemical or extract should be treated as a potential health hazard, and exposure to these chemicals or extracts should be minimized. Each laboratory is responsible for maintaining awareness of OSHA (1976) regulations regarding the safe handling of chemicals used in this method.

5.2 It is generally recommended that the laboratory personnel avoid contact with air particulates and that all operation steps, such as Soxhlet extraction or sonication, solvent evaporation, solvent exchange with dimethyl sulfoxide (DMSO), etc., be carried out in a hood (A-type). Hot water baths heated on electric heating mantles are recommended for solvent evaporation.

6.0 Apparatus and Equipment

A general list of the apparatus and equipment necessary for air sample preparation is presented below. For many items, equivalent products are available from other sources. Listing does not constitute a specific endorsement.

6.1 Sampling--

6.1.1 High-Volume (Hi-Vol) particulate sampler (Sierra Instruments, Model 305-2000).

6.1.2 Glass-fiber filters (8 x 10 in or 20.3 x 25.4 cm EPA Grade, Whatman).

6.1.3 Pallflex TX40H120-WW Teflon-coated filters.

6.2 Extraction and Sample Preparation--

6.2.1 Soxhlet extractor: 40-mm ID, with 500-mL round-bottom flask.

6.2.2 Sonicator (Bransonic, Models 220 or 32).

6.2.3 Kuderna-Danish (K-D) evaporative concentrator with a 3-ball Snyder Column (Kontes, Vineland, New Jersey).

6.2.4 Rota-Vap evaporator (Kontes).

6.2.5 Heating mantles (Fisher Scientific Company, Scientific Products, Inc. or Kontes).

6.2.6 Lab Jack support platform (Fisher Scientific Company, Scientific Products, Inc. or Kontes).

6.3 Miscellaneous--

6.3.1 Glass beakers (Pyrex brand): 100-, 250-, 500- and 1,000-mL (Fisher Scientific Company).

6.3.2 Extract storage containers: 10-, 25- and 50-mL amber-colored bottles with polytetrafluoroethylene-lined screw caps (Fisher Scientific Company or Kontes).

6.3.3 Analytical balance capable of weighing 0.0001 g accurately (Mettler Instrument Corporation).

6.3.4 Glass rods and stainless steel spatulas (Fisher Scientific Company).

7.0 Reagents and Consumable Materials

7.1 Solvents--

If the laboratory intends to use the purchased solvents without redistilling, materials bearing the manufacturer's designation of "spectral-grade or pesticide quality or distilled in glass" should be purchased. If drum lots of technical- or commercial-grade solvents are bought, distillation through an all-glass still is practically mandatory.

Spectral-grade or pesticide quality solvents (from: Burdick and Jackson Laboratories or Fisher Scientific Company) include:

Dichloromethane (Methylene chloride)
Cyclohexane
Methanol
Dimethyl Sulfoxide (DMSO)

7.2 Disposable Items--

Screw-capped Teflon vials (Fisher Scientific Company or Kontes), Soxhlet extraction thimbles (Fisher Scientific Company or Kontes), 0.5-μm Fluoropore filters (Millipore Corporation) and latex surgical gloves (Pharma Seal Laboratories) are included among the disposable items to be used.

8.0 Sample Collection, Preservation and Handling

8.1 Sample Collection--

8.1.1 Source and ambient air particulate collection methods cover a wide variety of physical collection techniques. The choice of a sampling technique is usually source- or sample size-specific. Elaborate description of the various source and ambient air sampling systems is beyond the scope of this protocol. The reader is requested to refer to the relevant reference(s) in this section.

8.1.2 Source sampling--Source sampling techniques include application of impactors, cyclones, electrostatic precipitators and filter media to recover particulate emissions with or without the ability to select the particle sizes collected. Examples of source sampling systems include EPA Method 5, Source Assessment Sampling Systems, Instack Cyclone Systems and Dilution Tunnel Sampling. An overview of these sampling systems is briefly described in

IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Test (Brusick and Young 1981). A more detailed description is included in the IERL-RTP Procedures Manual: Level 1 Environmental Assessment (Second Edition) (Lentzen et al. 1978).

8.1.3 Ambient air sampling--Ambient air sampling techniques include the standard high-volume (Hi-Vol) samplers (Federal Register 1971), massive-volume samplers (Henry and Mitchell 1978), medium-volume samplers, low-volume samplers and ultra high-volume samplers (Fitz et al. 1983).

The sampling of POM in ambient air is usually performed with the objective of determining the particle size distribution and the nature and concentrations of individual components at various points in the environment. The selection of sampling sites plays an important role in sample collection. Sampling sites are determined to evaluate the following: (1) characterization of rural or urban background levels, (2) assessment of health hazards to people in the vicinity, (3) determination of source effects and (4) establishing of transport mechanisms. Reference should be made to the available sources (USEPA 1971, 1972) for detailed treatment of these aspects in the selection of sites.

In addition to the selection of a preferable method and site, certain other factors must be adequately taken into consideration during ambient sampling. The height of the sampler intake above ground level and the local topography and climate influence the data obtained. For example, the influence of summer-like temperature on losses of benzo(a)pyrene from airborne particles has been studied during real high-volume atmospheric samplings (DeWiest and Rondia 1976). Seasonal variation in the specific surface areas and densities of suspended particulate matter has been observed (Corn et al. 1971, Flessel et al. 1984). The effect of wind on the collection efficiencies of particulate matter has also been demonstrated (Ogden and Wood 1975). Other factors, such as sampling rate and time, are also important in the overall sampling strategy (Flessel et al. 1984; Sweetman et al., 1984). The total amount of samples to be collected usually depends on the amount necessary to perform the bioassay. The optimum amount of air particulates needed for both bioassay and chemical analyses is approximately 2 g. However, it is possible to do the Ames bioassay with 100 mg of the starting material.

For routine monitoring of the total mutagenicity of POM, air samples can be collected in standard Hi-Vol samplers on filters with inert filter (Teflon) collection media (Flessel et al. 1981, 1984, Sweetman et al. 1984).

Alfheim and Lindskog (1984) have recently compared different high-volume sampling systems for collection of POM for mutagenicity testing using Ames assay.

In certain instances, it is desirable to collect larger amounts of material for mutagen bioassay and detailed chemical analysis of the organic matter of the airborne particulate matter. In addition, short time collection intervals are also desirable to prevent evaporation or chemical reaction of organic compounds, some of which may be responsible for the positive response of the particulate extract in mutagen bioassay. The use of an ultrahigh-volume sampler developed by Fitz et al. (1983) will solve these problems, and it can also be used for monitoring the total mutagenicity of POM. However, it must be pointed out that the ultrahigh-volume sampler is not feasible for routine applications.

Since the method described in this protocol has been written using a standard ambient high-volume sample as a specific example, a brief description of this sampler is provided in this section.

8.1.4 Standard high-volume (Hi-Vol) sampler--The Hi-Vol sampler with a glass- fiber filter is one of the basic instruments for collecting total suspended particulate matter in the atmosphere and is simple in design and easy to operate (Air Quality Criteria for Particulate Matter 1969, Clements et al. 1972, Federal Register 1971, Jutze and Foster 1967).

8.1.4.1 High-volume sampler design--The standard Hi-Vol sampler, which is designated in the Federal Register (1971) as the official sampler for suspended particulates, used by the National Air Surveillance Networks (NASN), and included in the methods for atmospheric sampling recommended by the Air Pollution Control Association (Jutze and Foster 1967), has a peaked roof and orients the filter in the horizontal plane (Figures 5 and 6).

Air particulate matter is usually collected on flash-fired glass-fiber filters (preferably Teflon coated). The sampler consists of three units: (1) the face plate and gasket, (2) the filter adapter assembly and (3) the motor unit. The sampler must be capable of passing environmental air through an approximate 400-cm area of a clean 20.3 x 25.4 cm glass-fiber filter at a flow rate of 1.13 to 1.70 m/min (or 40 to 60 ft/min). The glass-fiber filters should have collection efficiencies of at least 99% for particles 0.3 μ or larger in diameter. The motor must be capable of continuous operation for at least 24-h periods. It is important that the sampler be properly installed in a suitable shelter to protect it from extreme weather conditions and debris. For measuring the air flow rates through the sampling unit, the sampler must be provided with either a calibrated flow meter (rotameter) or a gauge. The details of this method are given by Sholtes et al. (1970).

On the average, when a high-volume sampler is located in an urban area, it will collect approximately 250 to 350 mg of particulate matter while sampling 2,000 to 2,400 m^3 of air during a 24-h period. Approximately 10% (25 mg) will be the extractable organic fraction (Sawicki et al. 1970a, b, c). As mentioned earlier, the quantity of organic fraction generally needed

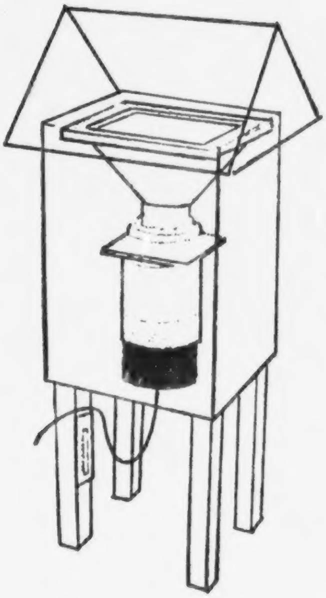


Figure 5. Assembled high-volume sampler and shelter (Federal Register 1971).

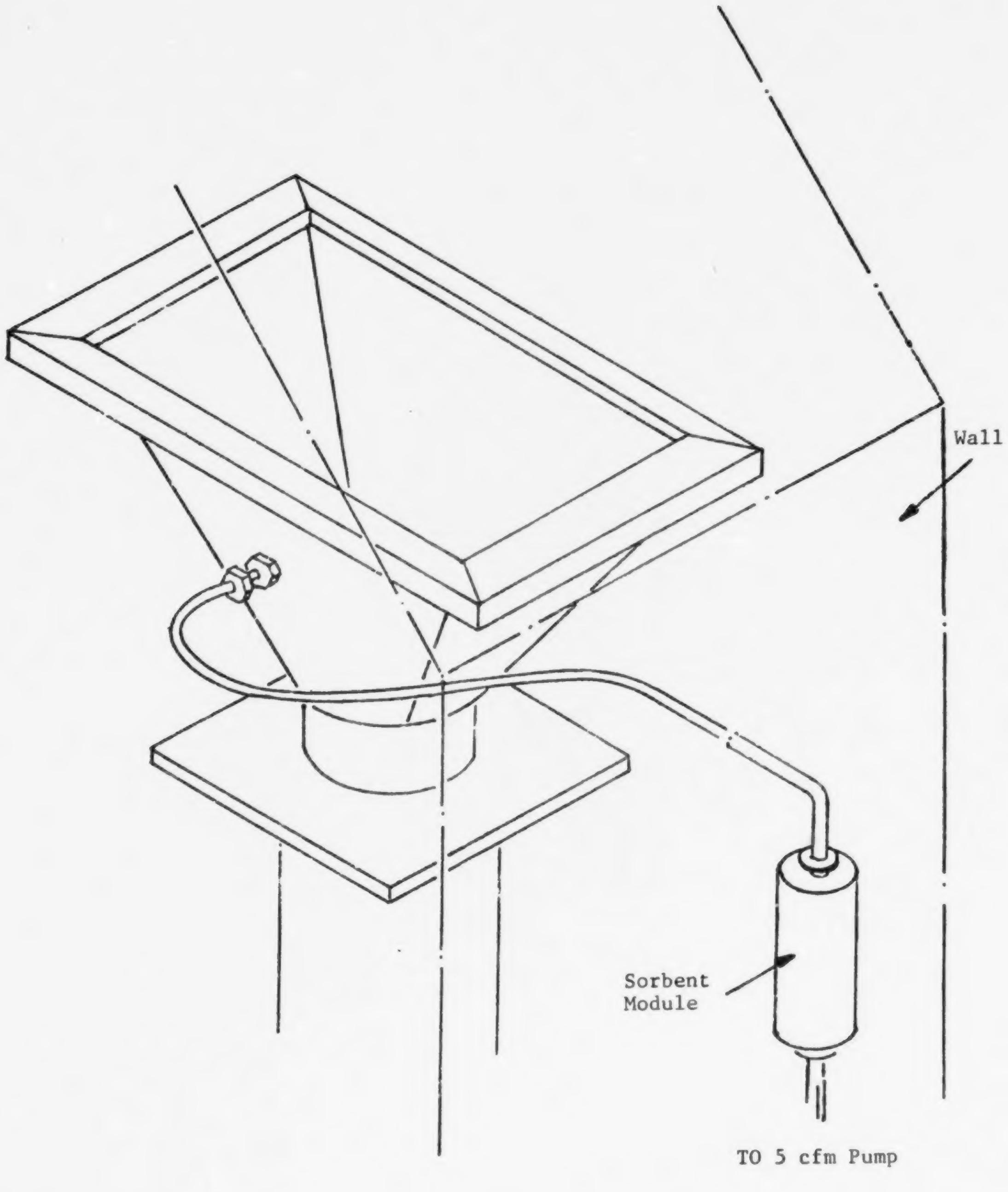

Wall
Sorbent Module
TO 5 cfm Pump

Figure 6. Diagrammatic presentation of connections for sorbent cartridge for high-volume sampler (Lentzen et al. 1978).

depends on the amount necessary to perform the bioassay. However, to obtain the needed amount of organic fraction, it may become necessary to pool together organic fractions of several individual high-volume air samples from a single monitoring site. Air should not be sampled for more than 24 hours in order to avoid artifacts due to chemical reactions occurring on the filters.

The POM present in polluted air sorbed onto airborne particulate matter is usually characterized as primary and secondary. Primary particulate matter is found in sizes between 1 and 20 μm (Fennelly 1975). Secondary particulate matter ranges in size from molecular clusters on the order of 5 nm to particles with diameters as large as several micrometers (Fennelly 1975). Very fine particles that cannot be retained on fiber-glass filters act like a gas or vapor in that they follow fluid streamlines and are subject to Brownian motion. Various absorbents have been used for collection of this gaseous phase (Bertsch et al. 1974, Fox and Staley 1976, Krstulovic et al. 1977, Schuetzle et al. 1973).

8.1.5 Distribution of particle size--An assessment of the POM content of the polluted atmosphere with respect to the size of particles in an aerosol often becomes necessary. Knowledge of the fraction of the air particulate matter which can cause deposition in the various compartments of the respiratory system is of prime importance since some of the POMs are proven carcinogens. Size fractionation of particles is an important factor for the quantitation of mutagenicity, since the smaller sized particles (≤ 2 μm) present a greater surface area for adsorption of the organic pollutants per unit mass of particulate. The smaller particles are more easily inhaled and deposited in the lung and are more difficult to expel (Hughes et al. 1980, Kolber et al. 1983, Lippmann et al. 1979, Natusch and Wallace 1974, Schlesinger and Lippmann 1978, Yeh et al. 1976). An increase in mutagenicity with decreasing particulate diameter (increased surface area) has been demonstrated for coal fly ash (Fisher et al. 1979) and for air particulates (Commoner et al. 1978).

Whitby et al. (1974) have presented evidence that the mass distribution of atmospheric aerosols is usually bimodal, with one mode occurring below 1.0 μm and the other mode occurring in the 5- to 15-μm range. A dichotomous sampler for particulates has been designed to collect and fractionate samples into two size ranges (Dzubay and Stevens 1975). Membrane filters in the two air paths collect the respective samples. However, such a sampling technique has rarely been applied to collect POM from air.

The experimental determination of particulate distribution is frequently done by five-stage Andersen Hi-Vol cascade impactors (Katz and Pierce 1976). The first four stages of the sampler constitute the fractionating head, while the fifth stage is a backup filter positioned between the fractionating head and a standard Hi-Vol air sampler. When operated at a flow rate of 20 cfm, the sampler fractionates suspended particulate matter into five aerodynamic size ranges according to certain cut-off diameters.

8.2 Preservation and Handling--

8.2.1 Air particulate samples--After sampling, filters are folded into quarters (sample touching sample), held in glass-lined envelopes and transported to the laboratory from the field in a sealed container to minimize loss of sample during transport and handling. The filters are equilibrated at laboratory temperature and humidity for at least four hours and are weighed on a microbalance in order to determine the weight of air particulates. The filters are then placed back in the sealed container and stored as particulates at -20 C until solvent-extracted.

8.2.2 Field blanks--In order to have field blanks, some additional filters are also taken to the field, subjected to the identical weather conditions (temperature, humidity, pressure, etc.), brought back to the laboratory in a sealed container and stored at -20 C until solvent-extracted.

8.2.3 Solvent extracts--All solvent (dichloromethane and DMSO) extracts should be stored in properly labeled Teflon screw-capped amber-colored bottles (25- or 50-mL size) at -20 C. It is highly recommended that the DMSO extracts be bioassayed within three weeks from the extraction date, since DMSO is known to react with several organic compounds. Also, it should be emphasized that the DMSO extract should never be used for chemical analyses.

8.2.4 Handling--All particulate samples and extracts should be handled with proper care, since they contain either mutagens or carcinogens. It is recommended that the personnel working with these materials wear proper gloves (disposable latex surgical gloves, Pharma Seal Laboratories) and carry out all extraction steps under a laboratory hood (A-type).

9.0 Calibration

No specific calibration procedure is required for the air matrix.

10.0 Quality Control

10.1 Sample Extraction and Concentration--

Field blanks are extracted and carried through extraction procedures concurrently with the sample. If the field blank gives more than the background revertants (statistically significant compared to solvent blanks) in the Ames bioassay, the air sampling and field blanks have to be repeated until the field blank gives no significant positive response in the bioassay.

11.0 Procedure

11.1 Introduction--

As Guerin et al. (1978) point out, a major consideration in the mutagenicity testing of complex chemical mixtures is that such mixtures contain chemicals of a wide variety of classes and an equally wide range of concentrations which are potentially capable of causing additive, antagonistic or synergistic responses in test organisms or at specific receptor sites. Such interactions may be a function of the combined dose of toxicants or of the inherent genetic susceptibility of a particular target organism. The preparation of complex mixtures for *in vitro* bioassay (e.g., Ames test) is complicated by at least two factors: (1) the relevance of the material applied to the test system and (2) the compatibility of the material with the test system

(Guerin et al. 1978). Chemical "relevance" is achieved when the test system is dosed with a material whose chemical composition mimics that which reaches the natural point of impact. Often, difficulties with "compatibility" are encountered when the material being bioassayed contains constituents which interfere with the test organisms' ability to respond to the effect of interest. High concentrations of mildly toxic constituents or small quantities of highly toxic constituents can sometimes mask the more subtle effect of mutagenic constituents. Any steps taken to remove toxic constituents in order to make the material compatible with the test system necessarily involve a change in the physical or chemical nature of the test material. Thus, the objective of selecting the best sample preparation protocol is to choose a procedure which prepares materials in a form suitable for biotesting with a minimal or at least interpretable impact on the relevance of the test material.

The method described in this procedure has been written using a standard ambient high-volume sample as a specific example. With suitable modification, this procedure is applicable to most other particulate sampling techniques. Modifications to these procedures may be necessitated if the sampler type or source characteristics are different from those encountered when high-volume samples are used to collect ambient particulates. These changes will usually involve modifications to handle a variety of sample sizes or particulate types.

A number of methods are available for the desorption of the collected air particulates from the high-volume filter. The three methods most commonly used are (1) extraction, (2) thermal desorption and (3) vacuum sublimation. Of these three, the most efficient and widely used method is the extraction technique. In the extraction method, collected air particulates are desorbed from the filtering medium by means of solvent(s). The extraction procedure can be subdivided into three steps: (1) solvent extraction, (2) solvent evaporation and (3) solvent exchange with DMSO for bioassay.

11.2 Solvent Extraction--

The principle behind solvent extraction is to dissolve the collected particulates from the filtering medium by digesting in a suitable solvent. The usual method involves refluxing of the filter in a Soxhlet extractor for a certain length of time. As discussed in the Review of the Literature section, a wide variety of solvents have been used, including acetone, benzene, cyclohexane, dichloromethane, chloroform, methanol, pentane, carbon disulfide and tetrahydrofuran. Of these, dichloromethane, cyclohexane and methanol are the highly recommended solvents. Either extraction by cyclohexane or dichloromethane alone or sequential extraction using cyclohexane followed by dichloromethane has been recommended. The third choice is extraction using a mixture of cyclohexane:dichloromethane:methanol (1:1:1 v/v). However, EPA recommends extraction using either cyclohexane or dichloromethane alone (Lentzen et al. 1978, Brusick and Young 1981). The solvent extraction procedure is described using dichloromethane as the solvent of choice. A summary flow scheme for the procedure was previously presented in Figure 4.

Note: It is essential that a choice between these solvent systems be made only after comparing the extraction efficiencies on a particular air particulate sample. Thus, a side-by-side evaluation is recommended.

11.3 Solvent Extraction Procedure--

The extraction can be done using either a Soxhlet extractor (Figure 7) or a sonicator.

11.3.1 Soxhlet extraction--The air filters (containing 1 g of particulates) are folded into a bundle to prevent loss of material during the extraction procedure and to allow it to be introduced into the extractor. The sample is placed in a precleaned (with dichloromethane) Soxhlet thimble, introduced into the Soxhlet extractor and twice extracted with dichloromethane (100 mL) for 16 hr (5-10 cycles/hr) at the boiling point of the solvent (40 C). After cooling the extracts to room temperature, the two extracts are combined and filtered through a 0.5-µm Fluoropore filter (Millipore) into a round-bottom flask (500 mL). The solvent extract is then concentrated using either a rotary evaporator apparatus or a Kuderna-Danish concentrator.

11.3.2 Sonication--The widely used Soxhlet extraction is time-consuming and decomposes some of the pollutants (Sawicki et al. 1970a, b, c). In order to avoid these errors, a mechanical disruption technique by ultrasonic waves has been developed by Golden and Sawicki (1975). In this procedure, the filters are cut into small pieces (1-cm area), placed in Teflon screw-capped glass tubes and extracted twice with dichloromethane in a sonicator (Bransonic Models 220 or 32) at 40 C for 10 min each time. Each dichloromethane extract is filtered through a 0.5-µm Fluoropore filter (Millipore) into a round-bottom flask for solvent evaporation.

The entire sonication procedure has been evaluated by Sawicki et al. (1975). The recovery of PAHs by the sonication method was found to be 95% to 98.2%. A comparison of the recovery of aromatic compounds between the sonication and Soxhlet method showed that the ultrasonic method extracted 49% of the total particulate matter collected on glass-fiber filters, with a relative standard deviation ± 1.33% compared to 30% for the Soxhlet extraction, with a relative standard deviation ± 26.1% of the total extractables. Not only the extraction efficiency but also the reproducibility of the ultrasonic method was superior to the Soxhlet method.

However, recent literature recommends both these techniques equally (Alfheim and Lindskog 1984, Hughes et al. 1980, Krishna et al. 1983b).

11.4 Solvent Evaporation--

It will usually be necessary to concentrate the extract of air particulates to a volume of 10 mL or less for subsequent analysis. It is recommended that concentration to slightly less than a 10-mL volume be accomplished using a Kuderna-Danish (K-D) apparatus (Figure 8) with a three-ball Snyder column for total volumes between 100 mL and 1 L. For volumes greater than 1 L, a rotary evaporator (operated at 30 to 35 C, using tap water aspirator for vacuum and circulating ice water through the condenser) should be used to reduce the initial volume to approximately 100 mL. The resulting extract may then be concentrated further by K-D. In order to prevent loss of material, it is essential that the extract not be reduced to dryness at this point in the preparation scheme (Brusick and Young 1981, Lentzen et al. 1978).

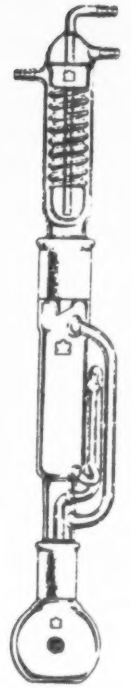
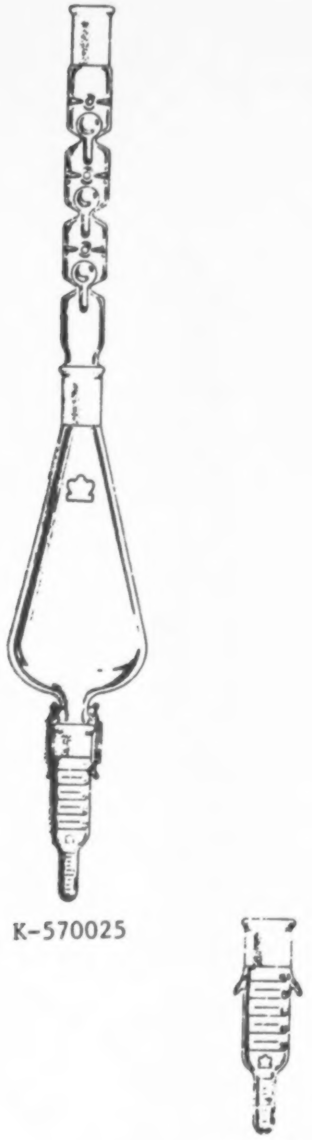

K-585250

Figure 7. Soxhlet extractor.



K-570025

K-570050

Figure 8. Kuderna-Danish evaporative concentrator.

An aliquot (up to 50% or 5 mL) of this extract should be set aside (in an amber-colored Teflon screw-capped bottle at -20 C) for mass determination (by gravimetric analysis) and chemical analyses or for retesting in the bioassay.

The remaining sample should then be transferred to a graduated container (e.g., K-D receiver or centrifuge type) and concentrated to a minimum of 1 mL or to the point where material begins to drop out of solution. In the latter case, the extract should be restored to a convenient volume in which the material is redissolved.

11.5 Solvent Exchange with DMSO--

Bioassay testing requires that the dichloromethane solvent be eliminated (reduced to less than 1%) before the sample extract is applied to the test system. An appropriate amount of extract is carefully reduced to 1 mL at 40 C under a gentle stream of purified nitrogen. The solvent evaporates rapidly, so it is important that this operation be done under constant surveillance to ensure that the volume is not reduced below 1 mL. It is also necessary to warm the samples slightly, preferably in a water bath (40 C), to prevent condensation of atmospheric moisture in the sample caused by evaporative cooling.

The sample container is held between 35-40 C in a waterbath. One milliliter of DMSO is added and mixed by gentle agitation. The volume is reduced to a total of slightly more than 1 mL by bubbling purified nitrogen through the sample using a clean Pasteur pipet. Another 1 mL of DMSO is added and mixed, and the volume is reduced to approximately 2 mL. The sample is checked for residual methylene chloride (Brusick and Young, 1981, Lentzen et al. 1978), and nitrogen purging is continued until the residual methylene chloride is below 1%. The final volume of this DMSO solution is noted. The sample may then be diluted to the appropriate concentration (1 to 10 mg/mL) for Ames testing.

Note: In the above description steps, it is recommended that a comparison be made between with/without K-D and with/without solvent exchange steps.

11.6 Mass Determination by Gravimetric (GRAV) Analysis--

The gravimetric analysis is used for mass determination of extractables with boiling points higher than 300 C. This analysis should be done after the sample extract has been concentrated, since weighing at least 10 mg of sample is recommended in a gravimetric analysis, when possible. Weighing to a precision of ± 0.1 mg is adequate for purposes of Level 1 analysis. Sample and tare weights should be obtained by drying to "constant weight" (± 0.1 mg) in a desiccator over silica gel or Drierite. In performing a gravimetric analysis on a large volume sample (i.e., >50 mL), no more than 5 mL of extract should be evaporated to dryness. For extracts concentrated to 10 mL, a 1-mL aliquot is taken for GRAV analysis. The GRAV results should be reported as one number for the entire sample (Lentzen et al. 1978).

12.0 Calculations

12.1 From the slope of the dose-response curve (from the Ames assay) for a particular strain of bacteria (e.g., TA98), one can calculate the following in steps:

a. Net revertants/volume of DMSO extract

b. Net revertants/mass of material in the total volume of the extract

c. Net revertants/total mass of particulate taken for extraction

d. Net revertants/volume of air collected in 24 hours

e. Net revertants/m^3 of air, which is defined as the mutagenic density.

12.2 Illustration--

The calculation steps can be illustrated by the following example. The following assumptions are made in the calculation: Volume of air collected (using Hi-Vol sampler) in 24 hours = 2,000 m^3. Weight of particulate = 100 μg/m. Number of net revertants using a particular bacterial strain = 2 revertants/μg.

a. Amount of total suspended particulate (TSP) =

$$2{,}000\ m^3 \times \frac{1{,}000\ \mu g}{m^3} = 2{,}000\ \mu g = 200\ mg$$

b. Amount of extractable organics (assuming 10%) = 20 mg

c. Assuming a typical value of 2 revertants/μg:

2 rev./μg = 2,000 rev./mg
or = 40,000 rev./20 mg
or = 40,000 rev./2000 m^3 of air
or = 20 rev./m^3 of air

13.0 Precision and Accuracy

Precision and accuracy of the proposed protocol have to be determined by validation. The validation has to be carried out by taking known amounts of air sample, replicating the procedure (at least thrice) and testing the extracts in the Ames bioassay. The precision requirements should be defined by the goals of the procedure.

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SECTION 3

DRINKING WATER

REVIEW OF THE LITERATURE

Introduction

The presence of trace levels of organic compounds in drinking water has heightened the concern among the scientific community and throughout the public sector as to the possible human health impact of chronic exposure to these contaminants. An initial response to these concerns was the enactment of a series of Federal Acts, including the Safe Drinking Water Act of 1974, PL 93-523, and the Clean Water Act of 1977, PL 95-217. Although the implementation of this federal legislation has resulted in measurable improvements in the nation's drinking water in terms of the removal of toxic metals and the lowering of the concentrations of some organics such as trihalomethanes (CEQ 1979, 1980, 1981, 1982), "further studies of the identities, carcinogenicity, mutagenicity, mode of formation and practical methods of removal are needed for the organic contaminants in drinking water" (Crump and Guess 1980). A great variety of natural and synthetic chemicals find their way into both the surface and ground waters used as sources of drinking water. The chemical/physical properties of these compounds have allowed for measurable progress in some areas of improved water quality, e.g., detection and subsequent lowering of trihalomethanes, but there has been a lack of progress in other areas, e.g., isolation/identification and subsequent removal of mutagenic constituents in the nonvolatile residue organics.

In terms of chemical analysis, this broad spectrum of organic chemicals in water is divided into two categories: (1) compounds with low solubility and a volatility sufficient for rapid separation and identification by gas chromatography/mass spectrometry and (2) relatively soluble compounds with low volatility, which are not readily separated by purge/extraction techniques, but are collected by more extensive extraction/concentration techniques from water as complex mixtures of residue organics. These two categories contain approximately 10% and 90%, respectively, of the organics in drinking water (Garrison 1977). As noted in a U.S. National Academy of Sciences - National Research Council Report (NAS-NRC 1977), all but about 10% of the organic compounds in the first category have been identified and can be quantitated. This progress has been facilitated by the development of methods to isolate and quantitate these compounds, as discussed in the following selected reviews (Smith 1978, Nunez et al. 1984), compendia (Keith 1976, 1981) and detailed procedures (44 FR 69464, December 3, 1979, Coleman et al. 1981). Some of the identified organics in this category have been assessed as to their toxicological significance (NAS-NRC 1977, 1980a, b, 1982a, 1983), and the evaluation of others is in progress (USEPA 1983). However, studies focused on the isolation, identification, quantitation and toxicological assessment of individual compounds in the latter category of nonvolatile/non-purgeable residue organics in drinking water have been far more difficult. Several procedures, reviewed by Kopfler (1980) and Jolley (1981), have been proposed to isolate these residue organics from drinking water for biological assessment of their toxicological effects and for compound isolation/identification. Additionally, these procedures have been evaluated in a recent report, "Quality Criteria For Water Reuse" (NAS-NRC 1982b). The salient features of these procedures are discussed in the following sections. Also, several of these procedures have been applied to a variety of drinking waters for the isolation of residue organics to conduct toxicological assessment. Summaries of these studies in terms of short-term mutagenicity assays have been reviewed by Loper (1980), Kool et al. (1982) and Hoffmann (1982).

Overview of Procedures for Isolating Residue Organics

Any sampling method for obtaining the organics from dilute aqueous solutions should be reasonably quantitative and free of artifacts of isolation. The desired strategy would apply broad-range recovery methods which result in the co-concentration of the thousands of compounds in drinking water into a representative complex mixture of the residue organics. Since the great majority of these compounds are unknown and are present in only dilute solution, it is impossible to test the absolute efficiency of any one method.

The strategies and methods employed in sampling for the residue organics in water have been reviewed (Kopfler 1980, Jolley 1981, NAS-NRC 1982b), were discussed in the 1980 Symposium on "Concentrating Organics for Toxicity Testing" at the 179th American Chemical Society Meeting (ACS 1980), and were discussed further at the 1984 Symposium on "Concentration Techniques for Collection and Analysis of Organic Chemicals for Biological Testing of Environmental Samples" at the 188th American Chemical Society National Meeting (ACS 1984). The methods most widely used for isolation of the residue organics (Kopfler 1980) generally fall into two categories: (1) concentration methods, in which the water is removed from the sample by freezing-out, freeze-drying, evaporation, reverse osmosis or ultrafiltration and (2) isolation methods, in which the organics are removed from the water by extraction or by the chromatographic means of sorption/desorption using activated carbon or polar and/or nonpolar stationary phases. Different combinations of many of these methods have been used in the isolation of nonvolatile residue organics from drinking water in order to improve recoveries or process large sample volumes.

Methods Based on Water Removal--

The preparation of aqueous concentrates via water removal is limited by the concentration of inorganic substances in the sample and the aqueous solubility of the organic substances. As the water is removed, the inorganic species are concentrated along with the organics. Following the point of solution saturation, the inorganic and organic species coprecipitate, making recovery of the organic constituents of interest difficult. However, aqueous solutions concentrated to a point nearing saturation can be used directly for bioassay if the tester system has adequate sensitivity and the presence of inorganic salts does not interfere with the bioassay. In those situations where the salts interfere and/or the solution is still too dilute, the concentrates must be processed further by alternative methods such as extraction or chromatography. In general, the methods of freeze-concentration (Shapiro 1961, Malo 1967a, b, Baker 1970), freeze-drying or lyophilization (Dawson and Mopper 1978, Crathorne et al. 1979) and evaporation or distillation under reduced pressure (Jolley et al. 1975) have not been used for the preparation of residue organics from drinking water for bioassay due to the aforementioned limitations. However, these methods have been used for the preparation of residue organics from drinking water for chemical analysis (for example, see Buelow et al. 1973, Watta et al. 1982).

The concentration method of reverse osmosis has been used in the preparation of drinking water residue organics for bioassays. Although this method was originally reported by Hindin et al. (1969), further development was conducted by Deinzer et al. (1975) in order to concentrate trace organics from drinking water for compound identification by combined gas chromatography/mass spectrometry techniques. Further studies of the methodology by Kopfler et al. (1977) resulted in the development of a method that was successfully applied to six different U.S. drinking water supplies for the preparation of residue organics for toxicological assessment and compound identification. The method features partition of the reverse osmosis concentrates by extraction of the organics into petroleum ether, diethyl ether and acetone. Each reverse osmosis concentrate extract was initially shown to be mutagenic in spot tests by Simmons and Tardiff (1976). These preliminary studies prompted modification of the procedure to include the isolation of additional residue organics from the remaining aqueous concentrates via XAD-2 chromatography. The extracts and XAD-2 isolates were subsequently bioassayed in a variety of short-term tests by Loper et al. (1978), Lang et al. (1980) and Kurzepa et al. (1980) and shown to be mutagenic in bacterial test systems and to cause cellular transformation in mammalian cell lines.

The method of Kopfler et al. (1977) warrants a few additional comments. This reverse osmosis concentration system employs both cellulose acetate and nylon membranes for the concentration of organics. The two different types of membranes were used because of their more efficient selectivity in the rejection of specific classes of compounds: the former rejects ionized solutes and polar constituents, particularly hydroxy compounds, whereas the latter rejects nonpolar constituents, such as aromatic compounds. The recovery efficiency was high for the cellulose acetate membrane (93%), but efficiencies could not be calculated for the nylon membrane due to the low total organics concentrations in the influent. The overall efficiency of this method for the organics concentrated from the drinking waters of three of the six cities in the study ranged from 15% to 41%. However, one problem noted with this method was the leaching of a number of compounds from the membranes into the concentrates. A more significant problem with the reverse osmosis method was the accumulation of inorganic salts in the residue organics concentrate. Some organics were adsorbed to the salts, thereby making recovery difficult.

Methods Based on Isolation of Organics from the Water--

The preparation of extracts of residue organics by isolation techniques requires the use of solvents either in the direct liquid/liquid extraction of the drinking water or in the desorption of chromatographic stationary phases used to adsorb the organics from the drinking water. The solvent is the major limitation in the isolation techniques, in that impurities can be concentrated along with the organics being isolated. The most common sources of solvent impurities are the preservatives, usually antioxidants, that can react with sample components (Kopfler, 1980). Also, solvent impurities can interfere with subsequent bioassays of the isolated nonvolatile residue organics (Tabor and Loper, unpublished). The use of high purity solvents and the inclusion of solvent blanks is mandatory for all isolation techniques.

Solvent extraction techniques have had limited applications in the preparation of residue organics from drinking water for toxicological assessment. Two of the reasons for the lack of investigation of this procedure may be that the method is highly selective and is cumbersome, since large amounts of liquids have to be manipulated. However, the method has been investigated for use in the removal of organics from drinking water (Suffet et al. 1976) and has been employed to isolate organics from water for quantitation of contaminants (for example, Mieure and Dietrich 1973, Hites 1977). Additionally, it should be noted that Meier and Bishop (1984) recently reported the use of this method for the preparation of residue organics from wastewaters for mutagenic assessment.

The isolation technique developed most extensively is the use of activated carbon as a stationary phase in chromatography to adsorb organics from water passed through the column. The method has been used for decades to isolate organics from drinking water for compound identification and for water quality monitoring (Braus et al. 1951, Middleton et al. 1956, Rosen, 1976, McGuire and Suffet 1983). The method has been developed to process thousands of gallons of water to yield gram quantities of residue organics (Middleton et al. 1962). To isolate the residue organics, the activated carbon is extracted with chloroform and ethyl alcohol, in sequence, followed by solvent removal via evaporation. However, the recovery of organics from the activated carbon is not as reproducible as with other methods (Chriswell et al. 1977). More recently, the use of supercritical liquid carbon dioxide has been examined to extract the carbon (Modell et al. 1978). This extraction method may be useful not only because liquid carbon dioxide is a good solvent for several classes of organics, but also because the high temperatures needed for organic solvent extraction are not required. It should be noted that a previously undescribed potent mutagen was isolated (Tabor and Loper 1980) and identified (Tabor 1983) from a portion of a 125-gram sample of residue organics isolated from 50,000 gallons of finished drinking water via the "Megasampler" (Middleton et al. 1962).

Organic polymers have been used extensively as stationary phases for adsorbing organics from drinking water. The most widely used polymers (Kopfler 1980, Jolley 1981, NAS-NRC 1982b) are either the polystyrenedivinylbenzene copolymers or the polymethacrylate polymers, both commonly referred to as various XAD resins. The use of XAD resins for the concentration of nonvolatile residue organics from water was first described by Burnham et al. (1972). In this study, weak organic acids and bases and neutral organic compounds were adsorbed on XAD-2 and/or XAD-7 from water solutions at original concentrations in the parts-per-billion to parts-per-million range, some with about 100% efficiency. In a series of follow-up experiments (Junk et al.

1974), a larger number of compounds, spiked into water, were recovered from the XAD resins at efficiencies of 80% to 100%. These laboratory studies have been followed by other similar investigations. Malcolm et al. (1977) found that adsorption optimization requires control of both the flow rates and the pH of the sample. Furthermore, they estimated that an XAD-8 column could concentrate about 50% of the total organic carbon of the average water sample. The studies of Thurman et al. (1978, 1979) featured the use of XAD-8, a somewhat polar resin similar to XAD-7, and examined the efficiency and capacity of this resin for the adsorption of polar organics from water. One important result of the study was the derivation of an empirical relationship allowing for the matching of column sizes to sample size. In a more extensive study of XAD resins, van Rossum and Webb (1978) measured the recovery efficiencies of XAD-2, -4, -7 and -8 for organics from water. Additionally, they evaluated a variety of organic solvents for the elution of adsorbed compounds from the resins. Although they recommended the use of equal amounts of XAD-4 and XAD-8, Cheh et al. (1980) have reported that passage of water containing residual chlorine over XAD-4 yields mutagenic artifacts. Likewise, James et al. (1981) and Care et al. (1982) have reported the leaching of XAD resin components when the columns were eluted to recover residue organics from water. Therefore, it is imperative that XAD resins are cleaned extensively prior to use. Procedures for accomplishing this have been published (Junk et al. 1974, Webb 1975, USEPA 1978). Additionally, general studies of the chemistry of the XAD resins and their use as high performance liquid chromatography supports have been reported (Grieser and Pietrzyk 1973, Pietrzyk and Chu 1977a, b, Pietrzyk et al. 1978, Baum et al. 1979).

The XAD resins have been used extensively for the concentration of residue organics from drinking water for toxicological assessment. One reason for their wide use is based on the following argument of Yamasaki and Ames (1977). Since the XAD resins exhibit a high specificity for adsorption of apolar or lipophilic compounds of low polarity (Junk et al. 1974, Fritz 1979, Dressler 1979), organic compounds, which will exert biological effects and are thus capable of passing through biological membranes, are likely to fall in these categories. Glatz et al. (1978) and the Canadian research group (LeBel et al. 1979, Nestmann et al. 1979, Williams et al. 1982) utilized the nonpolar XAD-2 to prepare residue organics from drinking water for bioassay. One Dutch research group (Van der Gaag et al. 1982, Noordsij et al. 1983) utilized a series of three XAD-4 columns for the isolation of residue organics at pH 7, 2 and 9, respectively. Several research groups have used a combination of two different XAD resins for the isolation of nonvolatile residue organics. Another Dutch research group (Kool et al. 1981a, b, 1982, 1984, Zoetemann et al. 1982) utilized a combination of XAD-4 and XAD-8, whereas one U.S. research group (Loper et al. 1983, Loper et al. 1984, Tabor and Loper 1984) utilized a combination of XAD-2 and XAD-7. The XAD resins have been applied to waters other than drinking water for the isolation of residue organics for toxicological assessment. For leading references see Jolley (1981), Hoffman (1982), Maciorowski (1982) and Tabor et al. (1984).

Additional methods, employing not only XAD resins but also other stationary phases and/or techniques, have been investigated for use in concentrating residue organics from water. Carbridenc and Sidka (1979) have developed a combined solvent extraction/XAD procedure that was reported to give a total recovery of 88% of the organics. Drinking water extracts of residue organics prepared by this method were bioassayed for cytotoxicity and promotion. Most noteworthy is a multiple column technique developed by the Los Angeles County Sanitation District research group (Baird et al. 1980, 1981, Jenkins et al. 1983, Nellor et al. 1984). The method features a high-volume system made up of four sequential chromatography columns packed with microparticulate resins consisting of an anion exchange resin, cation exchange resin, XAD-2 resin and XAD-7 resin, and was shown to remove 85% to 90% of the total organic carbon from the water. This system was developed for use in the concentration of residue organics from drinking water, river water, storm runoff and reclaimed wastewaters for toxicological assessment. The other noteworthy method, that utilizes more than one stationary phase is the "parfait" column procedure developed by Johnston (Johnston and Herron 1979, Johnston and Verdeyen 1981) for adsorbing drinking water organics. In the original method, the column was packed in distinct layers with three different stationary phases: silica gel, a cation exchange resin and an anion exchange resin. Following passage of the water through the column, the effluent was evaporated under vacuum to concentrate the hydrophilic organics. Substances absorbed on the column were extracted by separating the layers in the column and eluted by buffer followed by acetone. Residue organics isolated by this procedure have been assessed for mutagenicity. However, in subsequent studies it was shown that the procedure was not practical because attempts to separate salts and organic compounds in the eluates were unsuccessful (Johnston, unpublished).

Recommended Procedure for Isolating Residue Organics

The goal of any method for the isolation of residue organics from drinking water is the preparation of a complex mixture of the residue organics that is reasonably quantitative, representative of the nonvolatile organics in the original water sample and suitable for bioassay and/or toxicological assessment. To be widely applicable, the method should meet several criteria, including: (1) the apparatus used should be reasonably portable, thus allowing for sample collection at a variety of sites and return to a central laboratory for processing; (2) the system should be relatively easy to use and operate, so that the laboratory technician with an average background plus experience can successfully conduct a sampling and (3) the system should be constructed from commonly available items, or the system components should be readily available commercially at a reasonable cost.

Although no single method currently available meets all of the objectives of these criteria and their goal, the procedure described in the Protocol section closely approximates the objectives. The method was developed based upon information described in this review, and specifically upon reports by LeBel et al. (1979), Loper et al. (1983, 1984), Tabor and Loper (1984) and Nellor et al. (1984). This method provides for the isolation of residue organics from drinking water, purified from both surface and ground sources, that are suitable for mutagenicity testing (Loper et al. 1983, 1984, Tabor and Loper 1984). Similar methodology (e.g., Nellor et al. 1984) and modifications of the methodology (e.g., Tabor et al. 1984) have been used for the preparation of residue organics from wastewater for mutagenicity testing.

A few comments on the apparatus and methodology (which are described in the Protocol) are warranted. The system is constructed of stainless steel, thereby providing for a durable system. All fittings are of Swagelok design, thereby allowing for multiple reuse without leakage, etc. The column packings, resins or glass wool, are supported on both ends by stainless steel sintered frits. The system is adaptable to large or small sized samples under a variety of line pressure situations. Also, the use of in-line bacterial filters allows some time between sample collection and column elution.

As to the choice of resins, preliminary experiments (Van Rossum and Webb 1978, Loper et al. 1983, 1984, Tabor and Loper 1984, Kool et al. 1981a, b, 1982) have shown that the combination of XAD-2 (or 4) and XAD-7 (or 8) will extract nonpolar and semipolar residue organics from dilute aqueous solutions like drinking water. Furthermore, the extracted organics are readily eluted from the resins. Although numerous solvent systems have been utilized for the elution of the residue organics from the XAD resins (Junk et al. 1974, Van Rossum and Webb 1978, LeBel et al. 1979, Baird et al. 1980, 1981, Van der Gaag 1983, Noordsij et al. 1983, Nellor et al. 1984), Tabor and Loper (1984) have shown the hexane:acetone system of LeBel et al. (1979) to be more suitable for extracting residue organics. Other solvent systems, e.g., diethyl ether or acetone followed by methylene chloride, gave either mutagenic artifacts or low yields in terms of recoverable mutagenesis. This latter study (Tabor and Loper 1984) established flow rates, sample volume limitations and general operation parameters for the system described in the Protocol section.

The procedure described in the Protocol may not recover the highly polar and ionic organic species along with the highly volatile, low molecular weight organics. As to the former category of organics, further studies of the systems of Baird (Baird et al. 1980, 1981, Jenkins et al. 1983, Nellor et al. 1984) or of the Dutch National Waterworks group (Van der Gaag et al. 1982, Noordsij et al. 1983) may provide a suitable method. The highly volatile organics can be quantitated via other methods (e.g., Coleman et al. 1981, 44 FR 69464, December 3, 1979). Alternatively, the system proposed by Van der Gaag et al. (1982) allows for the collection of the volatile organics at the same time as that of the residue organics. Although this system is not as portable as the one described in the Protocol, it is possible that the Dutch system will be developed further for future wide-range application and use.

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DRINKING WATER WORKGROUP REPORT

Workgroup Tasks

Primary Tasks--

During general sessions and workgroup discussions, three types of primary tasks were identified: (1) additions and/or modifications to the protocol, (2) specific study requirements to be written into any experimental design employing the protocol and (3) issues not addressed by the protocol that will require additional research. Examples and resolution of these types of primary tasks are summarized in the following sections.

Additions--One addition to the protocol was a series of definitions to clarify the scope of the protocol. The environmental medium, drinking water, has been defined as: water intended for human consumption. The compounds isolated via the protocol were termed "residue organics," defined as those compounds absorbed by XAD resins under the conditions described herein, and recovered by the solvent elution method of this protocol. The term "nonvolatile," in reference to the type of residue organics, was deleted from the protocol because of the vagueness of this term in describing the mixture of organics isolated via the method. Additional definitions were added for clarification.

One major addition to the protocol was the inclusion of methods for the cleaning of the XAD resins. The required quality control criteria and attendant methodologies have been included also. It was the consensus of the workgroup that this information was needed in order to more completely detail the protocol and to add a more uniform approach to the use of these resins.

A recommendation was added to the protocol that drinking water residue organics showing toxicity or a no-dose response on initial mutagenesis assays be separated via high performance liquid chromatography (HPLC) and the collected fractions of eluates be tested for mutagenicity. The methodology for this separation is based on the reports of Loper et al. (1983), Tabor and Loper (1980, 1984), Tabor et al. (1984) and Baird et al. (1980) and is detailed in the protocol attendant to the overall document on preparation of residue organics.

Modifications--A modification to the protocol was to change the flow rates for the passage of the water sample through the system. Originally, flow rates of 300 to 350 mL/min and 100 to 125 mL/min were recommended for the large-volume and small-volume collectors, respectively. These rates were modified to 250 mL/min and 100 mL/min for the respective collectors. Although data were presented to support the original flow rate values, it was the consensus of the workgroup that flow rates more closely approximating those recommended by the manufacturer (Rohm and Haas) should be employed.

Specific study requirements--Several questions and issues were raised in the general sessions that the Drinking Water Workgroup decided could be dealt with more appropriately as requirements of a specific study in which the protocol is employed. One question concerned the frequency of sampling for a given drinking water, and another concerned the number of parallel samples to be collected. The statistical design of a particular study requiring the use of the protocol will determine the frequency and number of samples needed to accomplish the objectives of a given project. However, it was recommended that a duplicate set of samples be taken as a minimum sample set in order to provide for cases where something may happen to one sample set.

Additional research needs--One issue not addressed by the protocol was the use of spikes and/or surrogates to assess operation efficiency for a particular drinking water sampling. A series of compounds needs to be chosen and evaluated for use as spikes and/or surrogates in the protocol.

A second related research need for this protocol is to characterize the system more completely as to recoveries of compounds from drinking water. Compounds used for this study should encompass a range of solubilities and chromatographic retention indices.

Due to the limitations of the XAD resins in terms of the types of organics (e.g., nonpolar versus polar) absorbed from dilute aqueous solution, additional investigations on the use of additional columns, e.g., MP-1 or MP-50, following the XAD columns are warranted.

Secondary Tasks--

A number of other questions were raised by the general group as to the use of this protocol. These tasks, assigned to the drinking water group, were discussed and the results are summarized below.

Type of water sample--Several questions were raised as to the type of water sample used in this protocol for the concentration of residue organics. In general, all types of waters, containing $\leq 5\%$ solids, can be used as samples for this protocol. However, the inclusion of a 25-cc bed volume column of Celite 545 in the system between the glass wool prefilter column and the bacterial filters is recommended for waters having a total organic carbon level greater than 20 ppm or containing particulate matter. A discussion of the inclusion of this column for wastewaters is contained in Tabor et al. (1984) and has been added to the protocol.

As to the type of disinfection used for drinking water treatment or to the source (ground versus surface) of drinking water, the protocol is viable for all of these types of finished drinking water.

Other issues from discussion of the air sampling protocol--A number of questions were raised during the discussion of the air sampling protocol that were to be considered by the drinking water group. The issues included: Volatile organics, solvent extraction, input requirements, definition of blanks, storage of samples, internal standards, solvent volume reduction, solvent purity and frequency of replicate analysis. These issues either have been addressed in the protocol or have been discussed in other sections of this summary.

Other secondary tasks--The other issues raised in the general group discussions have been addressed in the protocol or were discussed in other sections of this summary.

Key Peer Review Comments--

Several points were raised by the reviewers of the protocol and the attendant literature review. The major point was the need to adequately discuss the applicability and limitations of the protocol in terms of the types of residue organics concentrated from water by the method. This point has been addressed in both the Literature Review and Protocol sections.

A second point was the need to include methods for the cleaning of the XAD resins in the protocol. These methods have been added.

A third point was the need to include in the Literature Review a section detailing the rationale for the choice of the method for the protocol and the limitations of the procedure. This section was added to the review. Additional research needs for this protocol are summarized in this report.

A few questions were raised by the reviewers with regard to sample pH, flow rates through the collection system and choice of elution solvents. These issues were resolved in the drinking water group discussions, and appropriate changes were made to the protocol.

Summary of Drinking Water Workgroup Progress--

The tasks and issues summarized in the preceding sections of this report were discussed in the workgroup meetings, and two results were achieved. The first result was to make appropriate additions and/or modifications to the protocol in order to address the specific points raised in the discussion. The second result was to recommend additional research for issues not addressed by the protocol. The group was in unanimous agreement as to the results/recommendations of the group deliberations.

The group was also in unanimous agreement as to the final protocol and recommends its implementation as an interim method for the preparation of residue organics from drinking water for mutagenicity testing.

Summary of Key Workgroup Discussions

The major issues/tasks discussed by the workgroup were outlined in the preceding sections.

Major Consensus Opinions--

All issues/tasks discussed were resolved as noted in the preceding sections.

Unresolvable Issues--

There were no unresolvable issues in the drinking water workgroup.

Evaluation of Proposed Protocol

The protocol for the preparation of residue organics from drinking water for mutagenicity testing is the result of a number of investigations by various research groups in both North America and Europe. This protocol adequately addresses the task assigned to the drinking water group in providing a proven method for the isolation of residue organics from drinking water for mutagenicity testing. The method is applicable to the isolation of residue organics from drinking water purified from both surface and ground sources. The method provides for the reproducible qualitative recovery of residue organics from drinking water. The procedure may not recover the highly polar and ionic species along with the highly volatile, low molecular weight organics. With the inclusion of a 25-cc bed volume column of Celite 545, the protocol is applicable to wastewaters containing 5% solids. The method is restricted to use by, or under the supervision of, analysts experienced in chromatography and properly trained in the handling and use of biohazardous materials.

The protocol does not apply to those mutagens not absorbed on or not desorbed from, the XAD resins and other system components, e.g., glass wool. It is not applicable to the more volatile organics or to highly unstable mutagens which may be lost/destroyed/modified during the sampling or work-up procedures. It is not known what percentage of the total "mutagenicity" of a water sample is recovered by this protocol. However, data for multiple parallel samplings of a drinking water (Tabor and Loper 1984) indicate the precision of the method to be within the limits of the Ames test for the residue organics. There are some data (e.g., Kool et al. 1982a, 1984, Tabor and Loper 1984) to indicate that mutagenic artifacts are not formed by the chlorine residual in drinking water reacting with the system components (e.g., resins), but there is almost no data for other disinfectants which could be used (i.e., ozone, etc.) (Kool et al. 1981a, 1984, Zoetemann et al. 1982). The bioassay of the drinking water residue organics does not differentiate between mutagens present in the raw water and those formed during water treatment for the preparation of finished drinking water. The raw water data can be obtained via application of the wastewater protocol to the raw water.

In comparison to the five other media protocols, this protocol is applicable to aqueous samples derived from the wastewater protocol but is not applicable to the other four protocols.

The drinking water workgroup strongly and unanimously recommended that all workgroup reports be included as a separate review section in the final document of protocols. This is imperative, since these reports will present to the user not only the evaluations of the protocols but also the limitations and research needs for the protocols.

Other Data or Information Requirements

Information Gaps--

A few information gaps in this protocol were identified by the drinking water workgroup. Three specific gaps, each of which will require additional research, concern: (1) additional information to further characterize the system as to the recoveries of organic compounds with a wide range of polarities and solubilities; (2) information to determine the need to include ion exchange resin columns or other adsorbant columns in order to isolate ionic, polar species from water for mutagenicity testing and (3) investigations to determine the most suitable compounds to be used for spiking the water sample in the determination of recoveries from sample to sample. Each of these information gaps should be addressed in subsequent research efforts, and is discussed further in the following sections.

Research Program Needs--

Recovery studies--To date, most recovery studies of organics from XAD resins have focused on the recovery of nonpolar organic compounds. Studies are needed to better define the limitations of the XAD system in this protocol for recovering organics from dilute aqueous solutions. Two parameters should be kept in mind when choosing the organic compounds used in such a study: (1) the compounds should represent a wide range of polarities and (2) the compounds should represent a wide range of solubilities in water. One question to be answered in such a study is, "Where in the procedure do the losses occur?" Since irreversible binding to the resins may occur and/or the compounds may be too dilute in the effluent water from the system to be detected by conventional means, this question could be addressed by using radiolabeled compounds. The use of radiolabeled compounds will permit the accountability of the compounds through all components used in the protocol.

An additional aspect to be considered in these studies would be the inclusion of known mutagenic compounds or compounds having structures, polarities and solubilities similar to known mutagens. Information from these latter compounds would assist in the studies required to address the research needs described previously.

Use of ion exchange resins--Data on the use of XAD resins suggest that these resins do not recover highly polar and ionic species from dilute aqueous solutions. This may not be significant in terms of mutagens, since the majority of the known mutagens are relatively nonpolar organics. However, there may be unknown polar, ionic compounds in drinking water that are mutagenic and would not be concentrated from water by the XAD resins. Therefore, the possible inclusion in the collection system of columns containing anion and cation exchange resins should be investigated. The logical point at which to include these columns would be following the XAD resins columns, since the XAD columns remove the nonpolar and semipolar organics. Therefore, the ion exchange columns would receive an influent water containing polar, ionic species. These studies should include the use of model compounds to determine recoveries, as previously described under "Recovery studies."

Compounds for use as sample spikes--The current data on the use of the system described in the protocol suggest that the method provides for the reproducible qualitative recovery of mutagenic residue organics from drinking water. However, the development of an adequate quality assurance plan for this protocol will require the inclusion of steps whereby recoveries and system performance from sample to sample can be monitored; this can be accomplished by spiking the sample with known compounds. The choice of compounds is open to discussion at this time. Part of the answer as to the type of compound(s) will come from the results of the research previously suggested under "Information Gaps." However, compounds representative of known mutagens should be considered.

PROTOCOL FOR THE PREPARATION OF DRINKING WATER FOR MUTAGENICITY TESTING

1.0 Scope and Application

1.1 This method is used for the isolation/preparation of residue organics from drinking water for mutagenicity testing. Drinking water is defined as water intended for human consumption.

1.2 This method is applicable to the isolation of residue organics from drinking waters purified from both surface and ground sources. The method may be applicable to other waters (e.g., see Wastewater Protocol).

1.3 The method provides for the reproducible qualitative recovery of mutagenic residue organics absorbed by XAD resins from drinking water. Residue organics are defined in Section 3.2.

1.4 This method does not provide information on volatile organics or on highly polar and/or ionic organics that may be present in the water sample.

1.5 The apparatus employed in this method is portable, and its use is straightforward. This allows for application to a wide variety of drinking water sources in the field, and thus does not require the transport of water samples to the laboratory.

1.6 This method is restricted to use by, or under the supervision of, analysts experienced in chromatography and properly trained in the handling and use of biohazardous materials.

2.0 Summary of Methods

2.1 A field duplicate sample of drinking water is passed through specially designed chromatography columns containing polystyrenedivinylbenzene copolymers and polymethacrylate polymer stationary phases. Following passage of the sample through the columns, the residue organics are eluted from the collection system components with organic solvents, the solvents are removed by evaporation and the remaining nonvolatile residue organics are stored under nitrogen until mutagenicity testing is conducted.

2.2 The method described in this protocol is based on reports by LeBel et al. (1979), Loper et al. (1983, 1984), Tabor and Loper (1984) Baird et al. (1981), Jenkins et al. (1983) and Nellor et al. (1984) for the isolation of residue organics from drinking water. For the purposes of this method, residue organics are those which are absorbed by resins under the conditions described herein and recovered by the solvent elution method of this protocol. The procedure may not recover the highly polar and ionic organic species or the highly volatile, low molecular weight organics. The design and testing of the large-volume, >50 L and small-volume, <50 L sampling apparatuses is given in Loper et al. (1982, 1984), Tabor and Loper (1984) and Nellor et al. (1984). These tests, for example, have shown that the large sampling apparatus is capable of accommodating 1,100 L of low total organic carbon water (i.e., drinking water), and that the small sampling apparatus is capable of accommodating more than 200 L of similar water, both without apparent breakthrough of mutagenic residue organics. Additionally, the passage of chlorinated, 2-ppm, ASTM Type I water through the system did not produce residue organics that gave positive results in the Salmonella mutagenicity test using strains TA98 and TA100 in the absence and presence of metabolic activation. These studies established operation parameters such as flow rates and line pressures. The results of these studies show that the method provides reproducible qualitative recoveries of mutagenic residue organics from a wide variety of drinking waters prepared from ground and surface sources.

3.0 Definitions

3.1 ASTM Type I Water--

The American Society for Testing Materials (ASTM) defines Type I water as having a maximum total matter of 0.1 mg/L, a maximum electrical conductivity at 25 C of 0.06 μmho/cm, a minimum electrical resistivity at 25 C of 16.67 Mohm·cm and a minimum color retention time for potassium permanganate of 60 min.

3.2 Drinking Water--

Water intended for human consumption.

3.3 Field Duplicate Samples--

Two samples taken at the same time and place, under identical circumstances, and treated exactly the same throughout field and laboratory procedures. Analysis of field duplicates indicates the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

3.4 Liter Equivalent--

The amount of residue organics concentrated from one liter of water.

3.5 Resin Blanks--

Two types of XAD resin blanks are required. The first, a chemical contamination blank, is determined on an extract of the resin via gas chromatography. The second, a mutagen contamination blank, is determined by mutagenesis assay of residue organics eluted from the resins following passage of ASTM Type I water through the assembled collection system.

3.6 Residue Organics--

Those organics adsorbed by XAD resins under the conditions described herein and recovered by the solvent elution method of this protocol.

3.7 Solvent Blank--

Elution solvents are concentrated 1,000 times, and the concentrates are bioassayed for mutagenesis.

4.0 Interferences

4.1 The polystyrenedivinylbenzene copolymer stationary phase, XAD-2, and the polymethacrylate polymer stationary phase, XAD-7, should be cleaned extensively prior to use according to the methods described in Section 7.1 and analyzed according to the methods in Section 9.2.

4.2 Interferences to the mutagenicity tests from elution solvents will vary from supplier to supplier and from grade to grade. Therefore, it is recommended that pesticide-grade or equivalent solvents are used and that appropriate tests of each lot of solvent are conducted according to the procedures described in Section 9.1.

4.3 Interferences to mutagenicity tests and subsequent chemical analyses from the apparatus can be avoided by using the stainless steel apparatus or its equivalent, described in Section 6.1, allowing the sample and solvents to come into contact only with properly cleaned glassware, described in Section 6.11, and utilizing only TFE tubing and TFE sealants where required.

5.0 Safety

5.1 The toxicity and carcinogenicity of the residue organic samples generated in this method have not been defined; however, each sample should be treated as a potential health hazard. Procedures for handling such materials have been described (USNCI 1981).

6.0 Apparatus and Equipment

6.1. Apparatus--

The general design of the sampling apparatus is shown in Figure 9, and specifics of the design are shown in Figure 10.

6.1.1 Gauge, 0-100 psi, stainless steel with TFE diaphragm (Veriflo Model IR-101S25DG or equivalent).

6.1.2 Flow control valve, one-way, needle, stainless steel (Whitey SS-IRM4-S4 or equivalent).

6.1.3 Bacterial filter holder--

6.1.3.1 For high total organic carbon (20 ppm) or particulate-laden water, a 142-mm sanitary filter holder, 316 stainless steel (Millipore YY30 142 36 or equivalent) fitted with 1/4-in stainless steel pipe to tube male couplings (Swagelok SS400-1-4-316 or equivalent).

6.1.3.2 For low total organic carbon (20 ppm) or low particulate-laden water, a 47-mm high-pressure filter holder, 316 stainless steel (Millipore XX45 047 00 or equivalent) fitted with 1/4-in stainless steel pipe to tube male couplings (Swagelok SS4DO-1-4-316 or equivalent).

6.1.4 Bacterial filters--

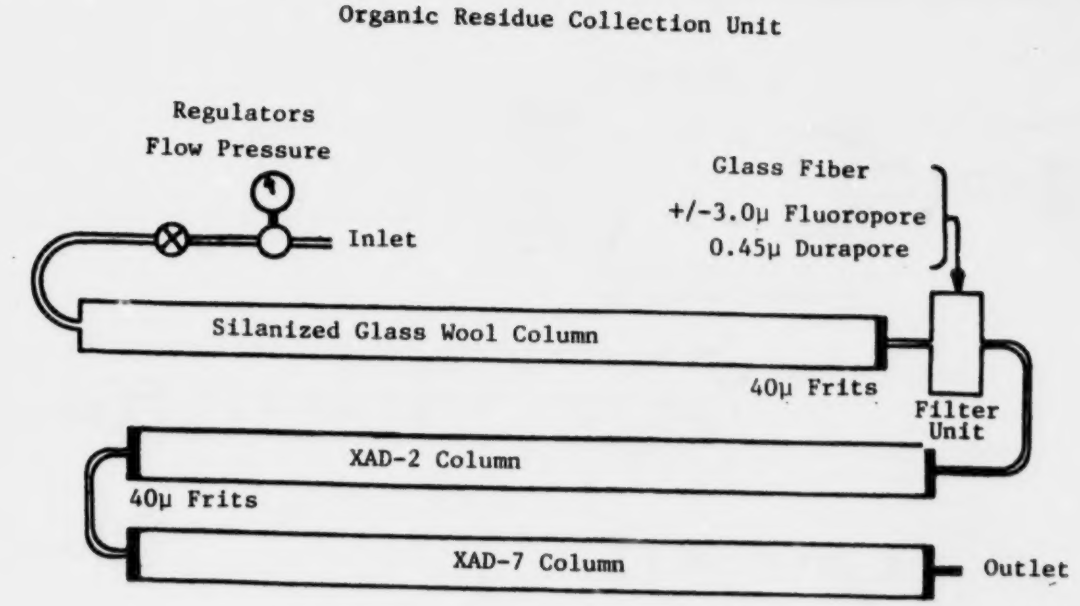
Organic Residue Collection Unit
Regulators
Flow Pressure
Inlet
Glass Fiber
+/-3.0µ Fluoropore
0.45µ Durapore
Silanized Glass Wool Column
40µ Frits
Filter Unit
XAD-2 Column
40µ Frits
XAD-7 Column
Outlet

Figure 9. Schematic of nonvolatile residue organics concentration apparatus.

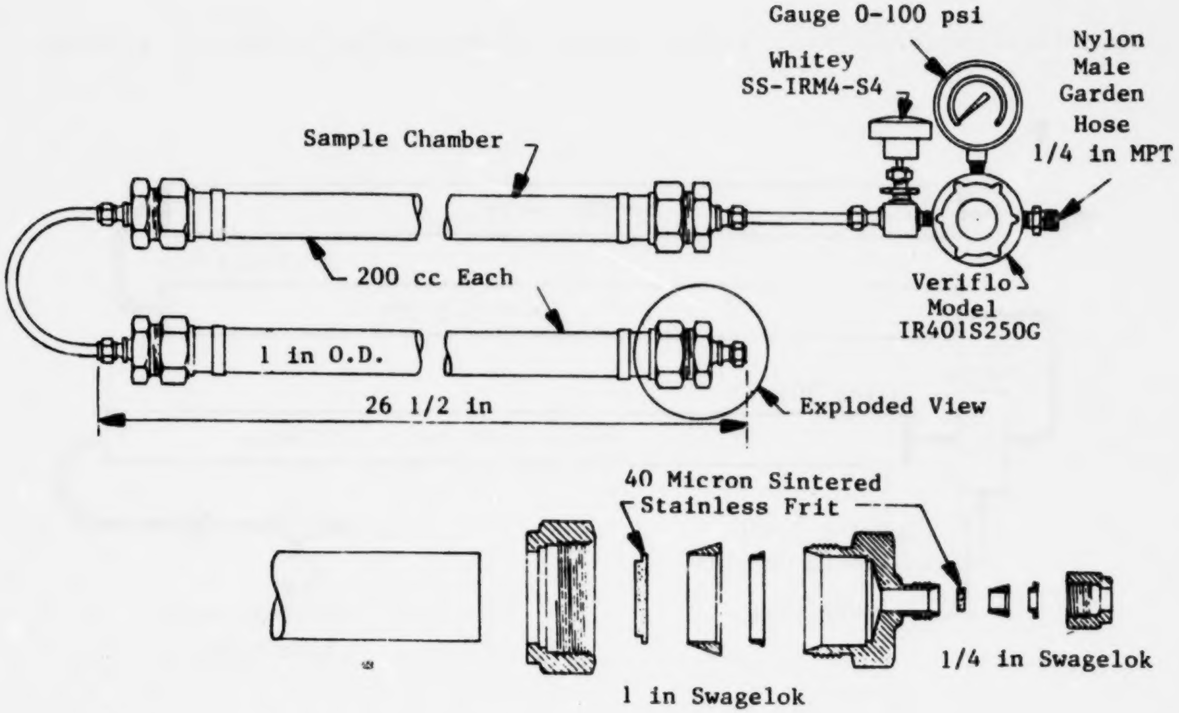
Gauge 0-100 psi
Whitey
SS-IRM4-S4
Nylon
Male
Garden
Hose
1/4 in MPT
Sample Chamber
200 cc Each
Veriflo
Model
IR401S250G
1 in O.D.
26 1/2 in
Exploded View
40 Micron Sintered
Stainless Frit
1/4 in Swagelok
1 in Swagelok

Figure 10. Details of nonvolatile residue organics concentration apparatus. (Note: prefiltration units are not shown.)

6.1.4.1 Microfilter glass discs without binder resin (Millipore Type AP40, AP40 047 05) for low total organic carbon filter holder.

6.1.4.2 Hydrophilic 0.45-micron Durapore filter (Millipore NVLP D4700 or equivalent) for low total organic carbon filter holder.

6.1.4.3 Hydrophilic 0.45-micron Durapore filter (Millipore HVLP 142 5D or equivalent) for high total organic carbon filter holder.

6.1.5 Connecting tubing, 316 stainless steel fitted with 1/4-in female Swagelok stainless steel fittings (Swagelok SS402-1-316, SS403-1-316, SS404-1-316 or equivalent). Any source of 316 stainless steel 1/4-in tubing acceptable.

6.1.6 Resin and glass wool columns--

6.1.6.1 Large sample volume (>50 L) columns of 200-cc bed volumes are constructed, as shown in Figure 10, of 1-in by 26.5-in 316 stainless steel tubing fitted with 1-in female Swagelok stainless steel fittings (Swagelok SS1012-1-316, SS1613-1-316, SS1614-1-316 or equivalent) and a 1-in male Swagelok stainless steel cap (Swagelok SS1610-C-316 or equivalent) modified as follows. The center of the 1-in cap is tapped and threaded to accept a 1/4-in pipe male coupling. The cap is fitted with a 1/4-in stainless steel pipe to tube male coupling (Swagelok SS400-1-4-316 or equivalent) which is silver-soldered in place. Prior to installation, the 1/4-in coupling is machined internally from the tubing side for an opening 3/16-in wide and 3/4-in deep. This opening is then fitted with a 5/32-in diameter by 1/8-in 40-micron sintered 316 stainless steel frit. Both column ends are fitted with the 40-micron sintered 316 stainless steel frits. This entire column assembly is available from Tristate Controls, Inc., 4303 Kellogg Avenue, Cincinnati, OH 45226.

6.1.6.2 Small sample volume (<50 L) columns of 25-cc bed volumes are constructed as shown in Figure 2 and described above, except that the columns are constructed of 1/2-in by 13-in 316 stainless steel tubing fitted with 1/2-in female Swagelok stainless steel fittings (Swagelok SS812-1-316, SS813-1-316, SS814-1-316 or equivalent), the 40-micron sintered 316 stainless steel frits and 1/2-in male Swagelok stainless steel caps (Swagelok SS810-C-316) modified as described above. This entire column system is available from Tristate Controls, Inc., 4303 Kellogg Avenue, Cincinnati, OH 45226.

6.2 Miscellaneous Apparatus Components--
(when line pressure of drinking water exceeds supplies 40 psi).

6.2.1 Pumping--The low line pressure drinking water sample source may be pumped through the columns by connecting a control volume TFE diaphragm pump (Milton Roy Co., model NR-117S or equivalent) between the drinking water outlet tap and the collection apparatus (Section 6.1.1). The pump should be capable of delivering 2.5 U.S. gal/h and developing a test pressure of 1,000 psi. The pump is fitted with a flow control valve, described in Section

6.1.2, to regulate flow rates. The pump is usually used when large sample volumes (>60 L) are to be collected.

6.2.2 Positive displacement--The low line pressure drinking water samples are collected in 20-L stainless steel reservoirs (Amicon Corporation model RS20 stainless steel reservoir or equivalent). The apparatus (Section 6.1.) is connected to the outlet of the reservoir by stainless steel tubing/ fittings. The inlet is connected to a nitrogen or helium gas cylinder fitted with a pressure regulator. The reservoir system is usually used when small volumes (<60 L) are to be collected.

6.3 Sample Storage Vials (Wheaton "500" amber serum bottles Nos. 223778, 223779, 223785 or 223787 or equivalent), sealable with Teflon-faced septa (Wheaton No. 224167 or 224172 or equivalent).

6.4 Resin Eluate Solvent Evaporator for initial reduction of volume (Buchi/Brinkman Rotary Evaporator, Model 15 00 500-9 or equivalent).

6.5 Sample Evaporator (Organomation Associates, Inc.) N-Evaps Model 111 or equivalent).

6.6 Analytical Balance - Readable to 0.01 mg with a precision of ± 0.01 mg.

6.7 Evaporation, displacement and sample storage gas, nitrogen or helium, water compressed, high-purity grade.

6.8 Special Glassware--

6.8.1 Evaporative concentrator, modified micro Snyder, 4-mL tube (Kontes No. K-569250 or equivalent).

6.8.2 Soxhlet extraction apparatus, Corning series 3840 or equivalent.

6.9 Muffle Furnace capable of sustaining 500 C, for use in glassware decontamination.

6.10 Solvent Reservoir 4-L stainless steel (Amicon Corporation, Model RS4 stainless steel reservoir or equivalent).

6.11 Flame Ionization Gas Chromatographic Unit fitted with a 6-ft by 4-mm glass column containing 10% OV-101 (or equivalent) on 100/120 mesh GAS-CHROM-Q. This unit must be capable of temperature programming.

6.12 Cleaning of Apparatus and Glassware--

6.12.1 Apparatus--Detergent wash, rinse with tap and ASTM Type I water, followed by successive rinses with pesticide-grade acetone and hexane.

6.12.2 Forty-micron sintered 316 stainless steel frits. Clean by sonication for 15 min in 6N nitric acid, followed by three successive 15-min sonications in ASTM Type I water. The frits are rinsed successively with pesticide-grade acetone and hexane.

6.12.3 Sample vials--Detergent wash, rinse with tap and ASTM Type I water, followed by drying/muffling overnight at 500 C. Tightly wrap vials in aluminum foil for storage until use.

6.12.4 Septum--Clean as for apparatus, Section 6.12.1.

6.12.5 General glassware--Clean as recommended in 44 FR 69464, December 3, 1979.

7.0 Reagents and Consumable Materials

7.1 Stationary Phases for Concentrating Nonvolatile Residue Organics--
Polystyrenedivinylbenzene copolymer, XAD-2, resin (Rohm and Haas Co.) and polymethacrylate polymer, XAD-7, resin (Rohm and Haas Co.) are available from numerous distributors. The XAD resins, as supplied, are contaminated with extractable monomeric and polymeric species that must be removed before use. The XAD-2 and XAD-7 resins are cleaned individually, but using the same procedure. The resin clean-up, detailed below, involves removal of fines, followed by Soxhlet extraction using a series of organic solvents. The purified resins are stored as an acetone slurry in amber bottles until use. Alternatively, the XAD resins, purified according to recommended methods and specifications, are available with certification of analysis from the Munhall Company, 5850 High Street, Worthington, OH 43085.

7.1.1 Resin cleaning--The resins can be cleaned in batches of 500 g, enough for one sampling plus required blanks, or multiples thereof. The following procedure is for 500-g batches, but can be scaled up as required.

7.1.1.1 To wash the resins and remove fines, transfer 500 g of the resin to a 1-L beaker and fill with ASTM Type I water. Slurry and allow to settle. Decant the supernatant fluid containing the fines and repeat until supernatant fluid is clear. This process may have to be repeated as many as ten times to obtain a clear supernatant fluid.

7.1.1.2 Following removal of the fines, the moist resin is transferred to a glass extraction thimble fitted with a fritted disc, and then the thimble is inserted into the Soxhlet extraction apparatus. For each 500 g of resin, the following extraction sequence is conducted using 1 L of solvent for each extraction. First the resin is extracted with 1 L of methanol for 8 h, followed by a 14-h extraction with an additional 1 L of fresh methanol. The 22-h methanol extraction is followed by extraction with two 1-L portions of methylene chloride, 8 h and 14 h, respectively. The 22-h methylene chloride extraction is followed by extraction with two 1-L portions of hexane, 8 h and 14 h, respectively. Finally, the resin is extracted with two 1-L portions of acetone, 8 h and 14 h, respectively. The resin is rinsed from the thimble into an amber bottle using a fresh portion of acetone. At this point, 20 cc of resin is taken for resin blank analysis, as described in Section 9.2 (see USEPA 1978). The resin is stored under acetone.

7.2 Glass Wool--
Silanized (Supelco No. 2-0411 or equivalent)

7.3 Solvents--

Methanol, methylene chloride, hexane and acetone, pesticide-grade or equivalent, and dimethylsulfoxide, DMSO, reagent-grade or equivalent, stored in original containers and used as received.

7.4 ASTM Type I water, generated by a Continental/Millipore Water Conditioning system (Tabor and Loper 1980) or equivalent.

7.5. Celite 545--

Prewashed as follows: Slurry 75 g of the filter aid in 500 mL ASTM Type I water by swirling, then settle briefly and decant the supernatant fluid containing the fine particles; repeat the process with a second aliquot of ASTM Type I water. Pack a 25-cc stainless steel column, fitted at the outlet end with a frit, with a 2-g plug of glass wool, then fill partly with ASTM Type I water. Slurry pack the column with the Celite 545, using Type I water as a liquid vehicle. Fit the inlet end of the column with a stainless steel frit, followed by the Swagelok fittings. Connect the inlet end of the column to a 4-L stainless steel reservoir (Amicon Corporation, model RS4 or equivalent). Successively wash the column with one liter each of pesticide-grade acetone, hexane and acetone, according to the procedure in Section 6.12.2. Following the last acetone wash, cap the column with 1/4-in stainless steel Swagelok plugs for storage of the column until use (within one week).

8.0 Residue Organics Isolation Procedure

8.1 Packing Procedure for 200-cc and 25-cc Columns--

The XAD-2 resin and XAD-7 resin columns are slurry packed, using acetone as a liquid vehicle. After packing, label flow direction and seal columns with 1/4-in stainless steel Swagelok plugs (Swagelok ss-400-P-316) for storage at 4 C until use (within one week). Do not allow the resins to dry; keep them covered with solvent. The glass wool prefiltration column is firmly but not tightly dry packed with 25 g of silanized glass wool, using a clean glass rod to position the packing material. Note: the stainless steel frits are omitted from the inlet end of the glass wool column. Label the flow direction of the column.

8.2 Apparatus for Drinking Water Sources with Line Pressure >40 psi--

The apparatus is assembled with proper flow directions for the columns, as shown in Figure 9, and is connected to the sample source via an appropriate fitting to the pressure regulator. When the Celite 545 column is required, e.g., for wastewaters, insert this column in-line between the glass wool column and the bacterial filter. When all of the filters and columns are connected in-line, turn on the water and partially open the valve to gently displace the acetone. Following the passage of one system volume of water through the apparatus, open the valve fully and adjust the flow rate. If using the large columns, set the regulator to 30 to 35 psi. For use of the large columns, adjust the flow rate to no greater than 250 mL/min by setting the pressure regulator to no greater than 30 psi (Tabor and Loper 1984, Loper et al. 1984). For use of the small columns, adjust the flow rate to no greater than 100 mL/min.

8.3 Apparatus for Drinking Water Sources at Line Pressures <40 psi--
The apparatus is assembled with proper flow directions for the columns, as shown in Figure 9.

8.3.1 Displacement of drinking water through the collection apparatus using nitrogen or helium--Connect the gas line to the inlet of a 20-L stainless steel reservoir containing the sample. Open the gas flow partially to gently displace the acetone. Following the passage of one system volume of water through the apparatus, further open the gas flow and adjust the tank pressure regulator to 30 psi. Flow rates can be regulated via the in-line valve. If the water sample is larger than 20 L or if the filters need to be changed, the flow may be stopped by closing the nitrogen tank and releasing the pressure via the pressure release valve on the reservoir. Following the required operation, changing of filters or connection to a new sample reservoir, close the pressure release valve and open the gas tank to resume flow; continue until all the sample has been displaced over the system.

8.3.2 Use of the in-line pump--The drinking water supply is connected to the pump inlet with appropriate fittings. This connecting line must be filled with water before the pump is turned on. The apparatus is connected to the pump, and the drinking water sample is pumped through the system. Note that a gentle flow of water is required in the beginning (Section 8.2) to displace the acetone.

8.4 Bacterial Filters--
When the flow rate decreases to approximately 50% of the initial rate, the bacterial filters probably need to be replaced. If the majority of the sample, >90%, has been concentrated, continue collection. If not, then discontinue the concentration operation, disassemble the bacterial filter holder and replace the filter(s) with fresh ones. The used filters are placed in amber bottles for storage until extraction. Handle these filters with forceps, since they are contaminated with bacteria, etc.

8.5 Volume Measurement--
The flow from the apparatus is collected in an appropriate measuring container. Usually 55-gal drums or smaller containers are used. At the end of the collection process, the total collected volume is recorded.

8.6 At the completion of a collection, the apparatus is disassembled in the order from the XAD-7 column, the last component in the system, to the drinking water source. All columns are sealed with stainless steel Swagelok plugs (Swagelok ss-400-P-316). Bacterial filters are removed from the holder and stored in amber bottles until extraction. Water in the glass wool column is allowed to drain, and the column is plugged as with the XAD columns. Columns and filters are stored at 4 C until extraction, usually within 3 days. The system should not be subjected to extremes of pressure or temperature.

8.7 Extraction of Residue Organics--
Resin columns and other system components, i.e., glass wool and filters, are extracted with a hexane:acetone solvent system, 85:15 by volume, according to the methods of LeBel et al. (1979), Loper et al. (1983, 1984) and Tabor and Loper (1984).

8.7.1 XAD columns--The XAD columns are individually mounted in an upright position so that the effluent end of the column is at the top. The end plugs on the top of the columns are removed, and the column is immediately connected via the top fitting to a pressure reservoir containing four to eight column volumes of hexane:acetone, 85:15 by volume, solvent. Following removal of the caps from the bottom of the column (i.e., the original inlet end), a length, 6 to 10 in, of 1/4-in stainless steel tubing is connected immediately to the bottom, and the free end of this tube is inserted into a cleaned glass separatory funnel to collect the water in the column. Pressure is gently applied (1 to 3 psi) to the system, and the column is allowed to fill with solvent. After the solvent has filled the column, stop the flow for equilibration for 15 to 20 min. The eluate tube is removed from the separatory funnel and inserted into a cleaned glass receiver. The system is pressurized for a flow rate not greater than one column volume per 20 min. The aqueous sample in the separatory funnel is extracted three times with equal volumes of pesticide-grade methylene chloride. These extracts are added to the solvent eluates from the column. The solvent eluates from the column are collected, passed through a small column (1 cm by 3 cm for the 25-cc system and 1 cm by 10 cm for the 200-cc system) of anhydrous sodium sulfate, and the eluates are collected in a cleaned round-bottom flask for initial concentration via rotary evaporation.

8.7.2 Glass wool columns--The columns are mounted upright and connected to a solvent reservoir containing four column volumes of hexane:acetone, 85:15 by volume, solvent. The top of the column is fitted to a tube in a manner similar to the XAD columns. The column is filled with solvent, which is allowed to equilibrate for 20 min. Following the equilibration, the solvent is replaced with fresh solvent, and the process is repeated. The extracts are combined for concentration via rotary evaporation. The glass wool is removed for further extraction. This is accomplished by Soxhlet extraction for 10 h using a ratio of five volumes of the hexane:acetone solvent for each cc volume of glass wool. The Soxhlet extracts are dried as before (Section 8.7.1) and are reduced in volume by rotary evaporation.

8.7.3 Bacterial filters--Each filter change group is extracted with 100 ml of the hexane:acetone solvent by soaking the filter in the solvent contained in a beaker for 20 min. This process is repeated two more times. The combined extracts are reduced in volume by rotary evaporation.

8.8 Concentration of Reduced Volume Extracts--

Following rotary evaporation, the volume of each extract is measured and recorded. The volume of each extract is reduced further using the micro-Snyder apparatus. Usually a measured aliquot of an extract is concentrated at one time, rather than concentrating the whole extract. The sample is gently heated using the N-Evap(a) bath. As the solution is concentrated, some constituents may come out of solution. If so, a small volume of acetone may be added to keep the components in solution until sufficient evaporation of the solvent has occurred to azeotrope the remaining hexane from the sample.

(a) Registered trademark.

Usually three to four additions of acetone are required. Final volumes of the acetone concentrates of the residue organic samples are recorded, and these samples are stored in Teflon-capped amber vials at -20 C until mutagenicity testing. At the time of bioassay, an aliquot of the residue solution is removed from the sample vial; typically, this aliquot is adjusted to necessary bioassay volume with DMSO. If it is necessary to know the mass of residue organics per dose, the amount of organics should be determined gravimetrically on a separate aliquot of the acetone solution of residue organics.

9.0 Quality Control

9.1 Solvent Blanks--

Samples of each lot of the hexane and acetone elution solvents and the methylene chloride extraction solvent are concentrated for mutagenesis testing. Two liters of each solvent and 2 L of the 85:15 hexane:acetone solvent are reduced in volume via rotary evaporation to 20 mL. Each sample is further concentrated to 0.2 mL via the micro-Snyder evaporative concentrator. The residue concentrate is mixed with 400 µL of DMSO and submitted for mutagenicity testing, two doses in duplicate, with Salmonella tester strains TA98 and TA100, in the absence and presence of metabolic activation (Loper et al. 1982, 1984). If the mutagenic response for any of the bioassay tests is equal to or greater than double the spontaneous rate, the solvent lot is rejected.

9.2 Resin Blanks--

9.2.1 Chemical contamination--The general procedure (USEPA 1978) for residual extractable organics is followed to determine contamination of the cleaned XAD resins. For each resin, a 20-g sample of resin is extracted for 22 h with 200 mL of acetone using a Soxhlet extractor. The 200-mL extracts are reduced in volume to 10 mL via evaporation under nitrogen, as described in Section 8.8. The concentrated extracts are analyzed by gas chromatography according to the USEPA total chromatographable organics analysis procedure (USEPA 1978). In this procedure, 5 µL of the extract are injected into a flame ionization gas chromatographic unit fitted with a 6-ft by 4-mm glass column containing 10% OV-101 (or equivalent) on 100/120 mesh GAS-CHROM Qm. Gas chromatography conditions: injector temperature 300 C, initial oven temperature 50 C, final oven temperature 250 C, temperature program rate 20 C/min, nitrogen carrier gas flowing at 40 mL/min, sensitivity 8 x, recorder range 1 mV. Resins are recleaned if the chromatograms of resin extracts show peaks greater than 10% full-scale eluting 5 min or later after injection.

The small sample volume apparatus (Section 6.1.6.2) is assembled as described. Two liters of the 85:15 hexane:acetone are eluted through the system and concentrated. Repeat this process until a gas chromatographic run of background is constant ±10%. Pass 1 L acetone through the columns, followed by 40 L ASTM Type I water. Elute columns per Section 8.7, and bioassay this residue. Mutagenic response of 1,000:1 concentrates should be less than 2x spontaneous reversion rate in the assay.

In cases where the organic residue is to be used for chemical analysis, it is desirable to characterize the solvent and water blank elutions by GC/MS or other specific analyses. In these cases, it is also recommended that chlorinated waters be dechlorinated with ferrous citrate (Cheh et al. 1979) prior to concentration in order to prevent resin artifacts from interfering with chemical analyses.

10.0 Sample Storage

10.1 Acetone concentrates of the residue organics are stored in Teflon-capped amber vials containing an inert gas (nitrogen or helium) atmosphere at -20 C until mutagenicity testing or further chemical analysis. The initial mutagenicity testing should be conducted within two weeks, since it has been noted that the bioactivity of some residue organics decreases with time (Loper and Tabor, unpublished).

10.2 If the initial bioassay of the residue organics results in toxicity or a no-dose response, a second portion (50- to 100-L equivalents) of residue organics is separated via HPLC, and the collected fractions of eluates are bioassayed for mutagenicity. The HPLC methodology is detailed in the protocol attendant to this overall document on the preparation of residue organics.

11.0 Data Records

11.1 Records to be maintained include a general description of the practices used in water treatment at the purification plant supplying the drinking water being sampled; time and location of sample collection; operation parameters for sample concentration, including flow rates, pressures, filter changes and volumes; details of resin preparation; data on quality control of resins and solvents; volumes of concentrated extracts and storage data and any unusual occurrences during the collection operation. Chain-of-custody forms should be executed for each sample from the time of preparation of the columns and of concentration of the water residue organics through the mutagenesis testing.

11.2 These records will be maintained for the glass wool extracts, the bacterial filter extracts, each XAD resin extract, the companion blank water system sample and each lot of solvents used in the extraction of a sample set.

12.0 Calculations

12.1 From data records, the final volume and/or weight of each concentrate is related directly back to the number of liters of drinking water concentrated. Mutagenesis data will be reported in terms of number of revertants or mutations per liter equivalent of water. Therefore, it is imperative that accurate records of all volumes be maintained throughout all operations.

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SECTION 4

ENVIRONMENTAL WATERS AND WASTEWATER

REVIEW OF THE LITERATURE

Introduction

Genotoxic agents are known to exist at very low concentrations in wastewater (Saxena and Schwartz 1979). However, mutagens, even potent ones, present at ppm and ppb levels may not be detectable when water samples are tested directly in sensitive bioassays such as the Ames test, since they fall below the sensitivity level for the bioassay. Consequently, special processing techniques are used to concentrate dilute wastewater samples before they are evaluated in such biological screens.

The rationale behind concentrating samples prior to subjecting them to bioassay procedures is that organisms in the aquatic food chain are known to bioconcentrate chemicals in a manner that might effectively yield hazardous levels of genotoxic agents when those organisms or animals which feed upon them are consumed by humans. A second reason is that long-term exposure of humans to very small quantities of water contaminants might result in high body burden if the chemicals are stored.

A primary objective in wastewater preparation and testing for biological activity is to collect samples that are representative of the waste effluent and to recover the bioactive molecules contained in the water in a state which retains their concentrations relative to each other. It is also important to prevent the formation of new combinations that might produce activity not initially present in the dilute wastewater.

Two approaches have been described for the processing of water samples for bioassay. The first involves concentration of all solubilized or suspended materials by removing only the water, and the second selectively removes the organic substances from the water. Examples of these two approaches, with their respective advantages and limitations are listed in Table 1. It is possible to combine methods which may improve the recovery of organic molecules from the water. Combination methods are useful when very large quantities of material (>50-L samples) need to be processed. Kopfler (1980) has described four methods which combine concentration and organic compound removal by ion exchange and/or XAD resins.

TABLE 1. METHODS PROPOSED FOR CONCENTRATION AND ISOLATION OF CHEMICALS IN DILUTE WATER SAMPLES

Method	Advantages	Limitations	Recovery	Method of Reference
Freeze concentration - a water sample is frozen into a shell of pure ice with the dissolved substances in the center unfrozen area.	Permits recovery of all dissolved materials including volatiles and ionized species.	Recovery of organic solutes decreases with increasing salt content of the samples due to alteration of ice formation. The level of concentration may be too low for recovery of very dilute species.	Evidence for up to 80% recovery of organics materials in a reconstruction experiment. Inorganic species will also be present in the concentrate.	Shapiro, J. 1961. Freezing out, a safe technique for concentration of dilute solutions. Science 133:2063-2064.
Freeze-drying - removal of water in vapor formed by freezing under vacuum. The solids suspended or solubilized in the water are left behind.	Recovers all species including organic and inorganic chemicals. Equipment is available to freeze dry large volumes (20-40 L) of collected water. The level of concentration is very high by the removal of all water.	Chemicals which are volatile at temperatures and pressures used will be lost. Since all dissolved chemicals will be recovered it is very difficult to remove the organics from inorganics. The process is time consuming.	Virtually 100% of all dissolved materials which are not volatile.	—
Reverse osmosis - water is removed by applied pressure across a membrane. The physical properties of the membrane determine the molecular weights of the materials remaining after the water is removed.	Commercial systems capable of handling large volumes are available. A large portion of ionic species and organic carbon species are retained in the concentrate.	The process can be time consuming. Since both inorganic and organic compounds are concentrated, a second process to separate these two classes would be required. Some commercial systems are not amenable to chlorinated water samples.	Recovery of 60%-80% of dissolved material is possible depending on the type membrane selected.	Kopfler et al. 1977. Extraction and identification of organic micropollutants: reverse osmosis method. Ann. N.Y. Acad. Sci., 298:20-30.
Direct liquid-liquid extraction - organics can be isolated from water by comixing immiscible organic solvents with wastewater. The organics will become solubilized in the organic solvent which can be easily separated from the mixture.	The process is direct and can be used with relatively large volumes (several liters). Reflexing of the solvent can concentrate the isolated organics into even smaller volumes.	Extraction of large volumes requires large amounts of solvent. The level of extraction is moderate since the process would require continuous removal and addition of fresh solvent to the water sample until all organics have been isolated. Concentration or organics by refluxing the solvent samples requires special equipment and may result in undesired chemical reactions during the heating process.	Not reported.	—
Isolation of organics on activated charcoal - water is passed over activated charcoal. The charcoal is then processed by Soxhlet extraction in organic solvents to recover organics.	The process perferentially concentrates organics on a solvent phase.	Carbon recovery is not as good as other solid phase media. The requirement for air drying and Soxhlet extraction with organic solvents might lead to artifact production.	Not reported.	Buelow et al. 1973. An improved method for determining organics by activated carbon adsorption and solvent extraction. J. Am. Water Works Assoc., 65:57-72.

continued-

TABLE 1 continued-

Method	Advantages	Limitations	Recovery	Method of Reference
Sorbant resin isolation of organics - this method utilizes a wide range of porous resins to recover organic compounds from water samples.	The resins commonly used (i.e., XAD-2 have great affinity for hydrophobic substances and retain virtually all of organic molecules. Once the water sample has passed over the resins, the trapped organics can be eluted from the resin. This process does not require Soxhlet extraction at high temperatures. The process can easily handle very large water samples and is relatively inexpensive.	Although resins are effective in adsorbing organic molecules, it is possible that not all organics of biological interest are adsorbed. If they are, it is possible that they cannot be eluted from the resin by the solvents used. Resin selection and preparation of columns for concentration.	50% to 90% of organics present in a sample can be recovered depending upon the parameters employed.	Junk et al. 1974. Use of macroreticular resins in the analysis of water for trace organic contaminants. J. Chromatogr., 99:745-762.

Objectives

The objective of this section is to review the methods for environmental waters and wastewater (referred to as wastewater) processing and recommend a method that will provide a convenient and reliable procedure to concentrate genotoxic chemicals suitable for Ames test evaluation.

The optimum methodology should meet a set of criteria proposed by Ross et al. (1980):

1. Concentrate relatively large samples.
2. Provide for a concentration factor $\geq$200 times.
3. Preserve volatile compounds.
4. Preferentially concentrate and extract potential genotoxic agents.
5. Reduce artifact formation.
6. Maintain relative concentration of all compounds.
7. Employ reagents that are compatible with bioassays.
8. Produce a relatively sterile test sample.

It is unlikely that any single method, or even combinations of methods, described in Table 1 would be able to meet all eight of these criteria; however, it should be possible to describe a method which achieves most of the desired properties.

Previous Studies

There are published reports describing water processing for various purposes, including the preparation of samples for bioassay (EPA 1977, Junk et al. 1974, Ross et al. 1980, Loper et al. 1978, Rappaport et al. 1979, Kopfler 1980). In virtually all of these methods, sorbent columns were used to concentrate the organic compounds which are the most likely candidates for genotoxicity. At least one of the studies (Ross et al. 1980) conducted tests for recovery of organic mutagens from spiked water samples as a means to estimate the reliability of the proposed techniques. In other studies, the methods were applied to actual wastewater samples collected from rivers and lakes. For example, pulp and paper mill effluents contain materials which are mutagenic in the Ames test (Nestman et al. 1979). The investigators in this study identified at least one mutagen, the resin neoabietic acid, in the effluents from a paper recycling plant. Other mutagens were obtained from solid waste collected from effluents emanating from a paper recycling plant (Klekowski and Levin 1979). In these studies, the mutagens could be collected as solids suspended in the water and did not require elaborate sample handling.

Follow-up studies to the wood pulp and paper making reports showed that chlorination of organic compounds derived from lignin stabilized their mutagenic activity (Nazar and Rapson 1980). Further studies along this line have shown that substitution of chlorine dioxide for aqueous chlorine or elevation of pH by adding lime to the treatment plant will substantially reduce the mutagenicity of wastewater (Nazar and Rapson 1980, Eriksson et al. 1979). In laboratory studies, Nazar and Napson (1982) showed that several Ames test mutagens produced during the bleaching of wood pulp (i.e., dichloroacetone, tetrachloroacetone and o-benzoquinone) could be inactivated by raising the pH of the treatment solution to pH 10 with NaOH. The mechanism appears to be by cleavage of organically bound chlorine by hydroxide ions.

In another study employing concentrations of 200-L samples of drinking water, river water and well water on XAD-2 resin columns, Nestmann et al. (1979), were able to demonstrate the presence of bacterial mutagens using strains TA-98 and TA-100. Bacterial mutagens have also been isolated from swimming pool water by Honer et al. (1980).

In a more recent study conducted on water samples collected from a lake in Illinois, the investigators identified mutagens active in strains TA-98 and TA-100 (Heartlein et al. 1981). The investigators in this study concentrated the water 3,000-fold on XAD-2 resin and performed preincubation tests with the eluents. The peak recovery of mutagens coincided with the application of pre-emergent herbicides used on nearby farms and probably involved leaching of herbicides or breakdown products from the fields into tributaries feeding the lake. Gas chromatography of selected lake water samples showed the presence of several pesticides, including some which are known to be mutagenic in *Salmonella*.

Chemical analysis of wastewater from trinitrotoluene (TNT) production suggests the presence of a large number of potential nitroaromatic mutagens. When the wastewater components were combined into a synthetic blend representing an average wastewater discharge, mutagenicity was observed using the Ames test (Spanggord et al. 1982).

In an unpublished report conducted under EPA contract by Litton Bionetics, Inc., eleven industrial wastewater samples obtained from selected outlets on the Chesapeake Bay (Table 2) were passed through the XAD-2 resin, and the organics were removed from the XAD-2 resin by Soxhlet extraction using acetone:dichloromethane. The acetone:dichloromethane extract was exchanged into dimethylsulfoxide (DMSO) and concentrated approximately 600-fold. The concentrated and unconcentrated samples were evaluated for their genetic activities in reverse mutation assays with *Salmonella* (Jagannath et al. 1981). Among the eleven samples, eight concentrates were clearly mutagenic in the reverse mutation assays with *Salmonella* strains TA-98 and/or TA-100. One sample was weakly mutagenic, and two were negative (Table 3). Column effluents and all of the unconcentrated samples were not mutagenic. Mutagenic activity showed up in samples with measurable levels of extractable organics, but there was no consistent relationship between the degree of mutagenicity and the amount of organic materials.

Recommendations

A comparison of the available methods against the optimal criteria for a sample processing procedure indicates that a method using sorbent resins followed by organic elution and concentration would probably come closest to the ideal system for routine wastewater processing for mutagenicity testing (Table 4). The available field testing results also support this recommendation.

TABLE 2. ORIGIN OF INDUSTRIAL WASTEWATER EFFLUENTS TESTED IN THE AMES ASSAY

Sample Number[a]	Industry Type	Raw Material	Product Description	Source of Water
100	Pulp and Paper	Wood fibers	Paper products, bleached wood	River
101	Plastics, Synthetic Fibers	--	--	Deep wells
102	Textile	Velveteen, corduroy, cotton, dyes, pigments	Finishing and dying of textiles	River
103	Petroleum Refining	Venezuelan and Mideast crude	Gasoline and heating oil	City supply and river water
104	Textile	Cotton, polyester, wool	Dying, finishing, printing fabrics	River
105	Paper Boar Mill	1000 TPD hardwood chips, NaOH, Na	Manufacture of corregated medium from pulp produced from non-sulfur, semi-chemical pulping process	River
106	Pulp and Paper	1200 TPD virgin material 130 TPD recycled hardwood, pine and corrugated paper	Unbleached craft paper corrugated medium, bleached market pulp	Wells
107	Synthetic Resins	--	Synthetic resins and fibers	Wells and springs
108	Tanning	131,000 cattle hides	Vegetable and mineral tanning of brine hides; curling finishing hides - leather converters	Wells, creek
109	Chemical Manufacturing	--	Manufacturing industrial inorganics and amines	River
110	Unknown	--	--	Unknown

(a) Each sample consisted of two gallons of wastewater.

TABLE 3. CORRELATION OF GENETIC ACTIVITY IN SALMONELLA ASSAY TO EXTRACTABLE ORGANICS FROM CHESAPEAKE BAY SAMPLES AND SPIKED SAMPLES

Sample	mg Organics/ mL Extract[(b)]	MEC (μg per Plate)[(c)]	Revertants per Plate[(d)]	Strain[(e)]	Specific Activities[(a)] Revertants per mg Organics
Spike[(f)]	0.063	1.575	510.0	TA-100	323809.5
100	1.600	320.000	241.0	TA-100	753.1
101	7.100	710.000	257.0	TA-100	361.9
102	0.500	12.500	198.0	TA-100	15840.0
103	1.900	95.000	213.0	TA-100	2242.1
104	0.200	20.000	42.5	TA-98	2125.0
105	1.000	50.000	72.5	TA-98	1450.0
106	1.300	130.000	34.5	TA-98	265.4
107	<0.100	20.000	31.5	TA-98	1575.0
108	0.100	>20.000	18.0	TA-98	1900.0
109	<0.100	>20.000	6.0	TA-98	300.0
110	0.900	>180.000	17.0	TA-98	94.4

(a) Revertants per plate (d) x 1,000 divided by MEC (c).

(b) Total organics extracted from one gallon of the sample. Final extract volume 10 mL.

(c) MEC: Minimum effective concentration which exhibited a minimum of two-fold increase in the number of revertants over the background.

(d) Revertants per plate in the presence of S-9 minus the spontaneous revertants.

(e) Strain from which the data was taken to extrapolate the specific activity.

(f) A one-gallon sample was spiked with 492 μg of N-nitroquinoline-N-oxide to serve as a known source of mutagens.

TABLE 4. COMPARISON OF AVAILABLE PROCESSING METHODS

Criteria	Freeze Concentration	Freeze-Drying	Reverse Osmosis	Direct Liquid-Liquid Extraction	Activated Charcoal Isolation	Sorbant Resin Isolation
Concentrate large samples	(a)	(b)	(c)	(a)	(b)	(c)
Provide $\geqq 200$ times concentration	(a)	(d)	(c)	(b)	(b)	(d)
Preserve volatiles	(c)	(a)	(c)	(b)	(b)	(b)
Preferentially concentrate genotoxicants	(a)	(a)	(b)	(c)	(c)	(d)
Reduce artifacts	(d)	(c)	(c)	(c)	(c)	(c)
Maintain relative concentrations of chemicals	(c)	(a)	(e)	(e)	(e)	(e)
Employ biocompatible reagents	(d)	(d)	(d)	(c)	(c)	(c)
Produce a sterile test sample	(b)	(a)	(b)	(c)	(c)	(c)

(a) Not a useful method.
(b) Poor method.
(c) Good method.
(d) Excellent method.
(e) Unknown.

Conclusions

Rapid inexpensive methods employing sorbent resins are readily available to capture organic compounds from wastewater samples of 3 to 20 L. Both laboratory and field studies have shown that these methods are capable of recovering mutagenic activity in a concentrated eluent from a sample which is not mutagenic in its neat form. With the exception of an organic extraction procedure employing dichloromethane (EPA Method 625, 1974), virtually none of the alternative methods have been adequately field tested. The EPA method has been used to prepare samples for Ames testing (D. Garnas, USEPA, NEIC, personal communication).

Application of XAD-2 resin column concentration of organics from aqueous samples, followed by solvent elution and concentration (200 to 1,000 times) in a biologically compatible solvent, can meet the objectives of the EPA for wastewater analysis and is recommended as the method of choice.

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ENVIRONMENTAL WATERS AND WASTEWATER WORKGROUP REPORT

Workgroup Tasks

Definitions of Wastewater and Wastewater Components--

The range of wastewater components spans from drinking water to nearly solids. Consequently, this spread must be divided into zones and components that can be processed.

Wastewater may contain suspended solids and nonaqueous liquids. These phases must be isolated and processed by independent methods.

The first task was to define the method of separating phases that might exist in a wastewater sample.

The second task was to prepare a standard method for gravity separation of the bulk sample.

The third task was to prepare a standard method for the determination of percentage suspended solids in the aqueous phase.

The fourth task was to prepare a standard method for liquid/liquid organic extraction of the aqueous phase containing suspended solids.

The fifth task was to propose standard methods for filtration and/or centrifugation of suspended solids from the aqueous phase containing suspended solids.

The sixth task was to propose standard quality control procedures for:

1. Sampling
2. Phase separation
3. Extraction and concentration
4. Bioassay

Peer Review Comments--

All peer review comments were incorporated into the final protocol.

Workgroup Progress--

a. Definitions

- Environmental waters and wastewater - nonpotable water containing less than 50% suspended solids by weight.

- Suspended solid phase - solid phase recoverable from the wastewater by gravity sedimentation, filtration or centrifugation.

- Suspended nonaqueous phase - suspended nonaqueous liquids.

b. Figure 11 defines how the collected sample will be separated into three phases and how these phases will be processed prior to bioassay.

Methods identified by footnotes are written elsewhere in this report.

Summary of Key Workgroup Discussions

Consensus Opinions--

Wastewater is a multiphasic medium which links naturally to other protocols.

Unresolved Issues--

a. Spiking of samples

  1. Examples
  2. When to add

b. Quantify mutagenic activity

  1. Revertants/mg total organics recovered from aqueous phase.
  2. Revertants/mg total organics recovered from nonaqueous liquid phase.
  3. Revertants/mg total organics recovered from suspended solid.
  4. Revertants/mg total organics recovered from sedimental solids.
  5. Revertants/mg total organics recovered from all sources.
  6. Revertants/liter of sample (unprocessed).
  7. Revertants/liter/hour of unprocessed emission.

c. Blanks - types, use, source, QC

d. TOC on initial sample?

e. Safety of methylene chloride washing

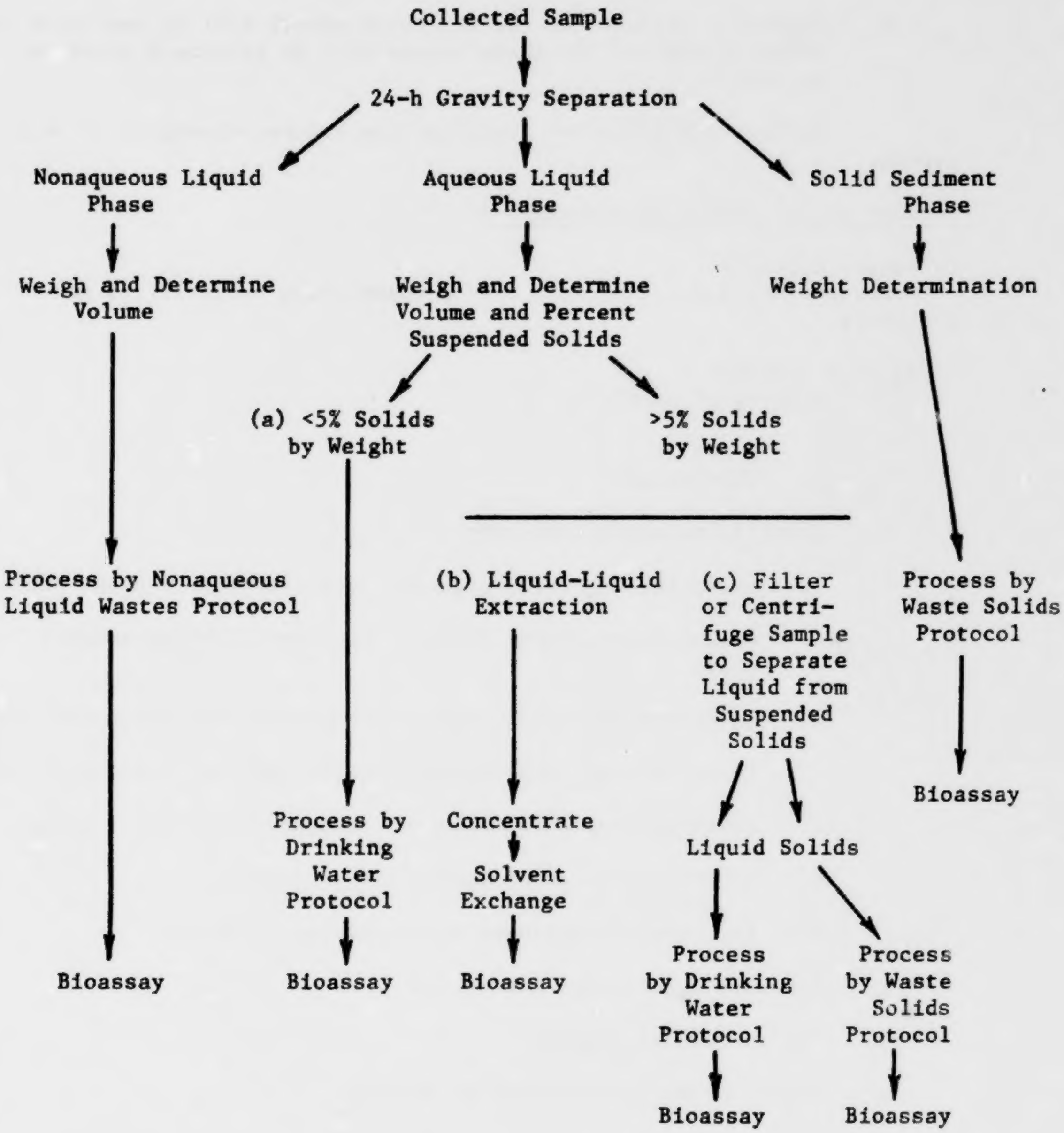
Evaluation of Proposed Protocol

A draft protocol was prepared and reviewed by members of the workgroup.

Other Data or Information Requirements

Information Needs--

a. Effects of matrix on mutagenic activity.


Collected Sample
24-h Gravity Separation
Nonaqueous Liquid Phase
Aqueous Liquid Phase
Solid Sediment Phase
Weigh and Determine Volume
Weigh and Determine Volume and Percent Suspended Solids
Weight Determination
(a) <5% Solids by Weight
>5% Solids by Weight
Process by Nonaqueous Liquid Wastes Protocol
(b) Liquid-Liquid Extraction
(c) Filter or Centrifuge Sample to Separate Liquid from Suspended Solids
Process by Waste Solids Protocol
Bioassay
Process by Drinking Water Protocol
Concentrate
Solvent Exchange
Liquid
Solids
Bioassay
Bioassay
Bioassay
Bioassay
Process by Drinking Water Protocol
Process by Waste Solids Protocol
Bioassay
Bioassay

Note: If results of (b) are negative (not mutagenic) it is recommended that the sample be subjected to (a) or (c) processing and retested in the bioassay.

Figure 11. Wastewater sample processing flow diagram.

b. Methods for bioactivity expression and what it means.

Other Needs--

a. Standard methods for sample collection, transport and storage - EPA group should be formed to do this.

b. How much chemistry data on samples should be developed?

PROTOCOL FOR THE PREPARATION OF ENVIRONMENTAL WATERS AND WASTEWATER FOR MUTAGENICITY TESTING

1.0 Scope and Application

1.1 This is a general purpose method which covers the preparation of wastewater samples for solvent-extractable organic compounds that are amenable to Ames mutagenicity assay.

1.2 The method is applicable only to solvent-extractable organic compounds, and not all chemicals in the sample are stable or recoverable by this procedure.

1.3 The final extract will be subjected to the Ames mutagenicity assay using the procedure of Williams and Preston (1982).

2.0 Summary of Method

Genotoxic activity of wastewater samples is assessed on the liquid and solid phases of wastewater grab samples. The discrete phases are collected, quantified, extracted and concentrated, and the resultant concentrates are transferred to DMSO and subjected to bioassay evaluation using a standard Ames test (Ames et al. 1975, Brusick and Young 1981, Williams and Preston 1982).

3.0 Definitions

Environmental Waters and Wastewater - Water not intended for human consumption, containing less than 50% suspended solids by weight, including water from industrial emissions, rivers, lakes and ponds. Wastewater may contain nonaqueous liquids in addition to the aqueous and solid phases.

Internal Standard - A pure compound added to a sample in known amounts and used to calibrate concentration measurements of other compounds that are sample components.

Field Duplicates - Two samples taken at the same time, placed under identical circumstances and treated exactly the same throughout field and laboratory procedures. Analysis of field duplicates indicates the precision associated with sample collection, preservation and storage, as well as with laboratory procedures.

Reagent Blank - Reagent water placed in a sample container in the laboratory and treated as a sample in all respects, including exposure to sampling site conditions, storage, preservation and all analytical procedures.

Nonaqueous Liquids Phase - Liquids whose major component is not water and which form discrete zones in an aqueous medium.

Sediment Solid Phase - The solid or semisolid phase recovered from a 24-h resting wastewater sample.

4.0 Interferences

4.1 Samples may change or become contaminated during transport from the collection site to the laboratory. Sample custody and shipping/storage conditions must be fully described and documented.

4.2 Emissions from wastewater sources change with time. Sufficient sample to complete all phases of the evaluation should be collected at the time of sampling. Date, time and exact location of sampling must be fully described and documented.

4.3 Method interferences may be caused by contaminants in solvents, reagents, glassware and other sample processing hardware that lead to discrete artifacts and/or elevated baselines in the total ion current profiles. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks.

4.4 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the industrial complex or municipality being sampled. An internal standard should be employed to evaluate matrix interference.

4.5 Methylene chloride can interfere with the mutagenicity assay, so care must be exercised to completely remove all methylene chloride prior to dilution with dimethyl sulfoxide.

4.6 The sample will be supplied for Ames testing in its most concentrated form in dimethyl sulfoxide, because additional sample concentration in the presence of dimethyl sulfoxide (B.P. 189 C) is prohibitive due to potential thermal or evaporative loss of sample organic constituents. If the extract is not soluble in its concentrated form, dilution with additional dimethyl sulfoxide or other appropriate solvents is advised.

4.7 Some extracted concentrated samples in DMSO may be highly cytotoxic in the Ames test. If a sample cannot be tested up to a level of 1,000 μg extracted organics per plate, it should be considered "toxic," and alternative treatment or bioassay methods should be performed, including chromatographic separation of toxic compounds from nontoxic compounds (HPLC or acid/base/neutral fractionation) (Hughes et al. 1980, Tabor and Loper 1980). In the case of limited sample size, the maximum level will depend only on sample availability.

5.0 Safety

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined; however, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available.

5.2 The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method (NIOSH 1977, OSHA 1976). A reference file of material data handling sheets should also be made available to all personnel involved in the chemical analysis. Additional references to laboratory safety are available and have been identified for the information of the analyst (ACS 1979).

5.3 Solvent rinsing of vessels with methylene chloride may result in excessive exposure to this solvent. All rinsing should be performed in an approved chemical fume hood, and appropriate personal safety apparel should be worn.

5.4 Internal control samples may be spiked with chemicals demonstrating carcinogenic activity in animals. Internal control samples should be handled according to safety procedures consistent with this fact (NCI 1981).

5.5 Care should be used when pressurizing or vacuum-filtering samples. Excessive pressure or vacuum might cause vessel or filter breakage and expulsion of concentrated sample into the laboratory environment.

5.6 Since some wastewater samples may come from sources containing human or domesticated animal excrement, communicable disease risk is present. Laboratory personnel should be adequately immunized for communicable diseases, and all samples should be considered potentially contaminated and handled accordingly.

6.0 Apparatus and Equipment

6.1 Grab Sample Bottle--

Amber glass wide-mouth, 3.8-L or 1-gal volume, fitted with screw caps lined with Teflon. Aluminum foil may be substituted for Teflon if the sample is not corrosive. Alternatively, Amicon stainless steel 20-L carboys may be used (see Drinking Water Protocol).

6.2 Pressure Filter--

Millipore unit fitted with a glass fiber filter (1-μ pore size).

6.3 Centrifuge--

High-speed, refrigerated sample centrifuge capable of handling 500-ml centrifuge tubes.

6.4 Celite column to trap particulate matter prior to resin concentration (acceptable devices are commercially available).

6.5 Glassware (all specifications are suggested. Catalog numbers are included for illustration only).

6.5.1 Separatory funnel--2,000 ml, with Teflon stopcock.

6.5.2 Drying column--9-mm ID chromatographic column with coarse frit.

6.5.3 Concentrator tube, Kuderna-Danish--25 mL, graduated (Kontes K-570050-2525 or equivalent). Calibration must be checked at the volumes employed in the test. Ground glass stopper is used to prevent evaporation of extracts.

6.5.4 Evaporative flask, Kuderna-Danish--500 mL (Kontes K-570001-0500 or equivalent). Attach to concentrator tube with springs.

6.5.5 Snyder column, Kuderna-Danish--Three-ball macro (Kontes K-503000-0121 or equivalent).

6.5.6 Vials--Amber glass, 10- to 5-mL capacity, with Teflon-lined screw cap.

6.5.7 Continuous liquid-liquid extractors--Equipped with Teflon or glass connecting joints and stopcocks requiring no lubrication. (Hershberg-Wolf Extractor-Ace Glass Company, Vineland, N.J. P/N 6B4-0 or equivalent).

6.6 Boiling Chips--

Approximately 10/40 mesh. Heat to 400 C for 30 min or Soxhlet extract with methylene chloride.

6.7 Water Bath--

Heated, with concentric ring cover, capable of temperature control (± 2 C). The bath should be used in a chemical fume hood.

6.8 Balance--

Analytical, capable of accurately weighing 0.0001 g.

6.9 Nitrogen Evaporator--

Equipped with Teflon or glass jets and temperature control. (Meyer N-evap Model 112 or equivalent - Organomation Assoc., Inc., Northborough, MA).

6.10 Standard amber glass storage containers, 10-mL bottles with Teflon-lined screw caps.

7.0 Reagents and Consumable Materials

7.1 Reagent Water--

Reagent water is defined as a water in which an interferent is not observed at the method detection limit of each parameter of interest. It consists of XAD-2 cleaned tap water.

7.2 Sodium Hydroxide Solution (10 N)--

Dissolve 40 g NaOH in reagent water and dilute to 100 mL.

7.3 Sodium Thiosulfate (ACS), granular.

7.4 Sulfuric Acid Solution (1+1)--
Slowly add 50 mL of H_2SO_4 (sp. gr. 1.84) to 50 mL of reagent water.

7.5 Acetone, methanol, methylene chloride, dimethyl sulfoxide (pesticide-quality or equivalent).

7.6 Sodium Sulfate (ACS) granular, anhydrous--
Purify by heating at 400 C for 4 h in a shallow tray.

7.7 Internal Standard Mutagen--
Use 4-nitroquinoline-N-oxide dissolved at 100 µg/L of liquid sample or other acceptable mutagens.

8.0 Quality Control

8.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

8.1.1 Before performing any analyses, the analyst must demonstrate the ability to generate acceptable accuracy and precision with this method.

8.1.2 In recognition of the rapid advances that are occurring in chromatography, the analyst is permitted certain options to improve the separations or lower the cost of measurements. Each time such modifications are made to the method, the analyst is required to repeat the procedure in Section 8.2.

8.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations.

8.2.1 Select a representative spike concentration for each parameter to be measured. Using stock standards, prepare a quality control check sample concentrate in acetone 1,000 times more concentrated than the selected concentrations. Quality control check sample concentrates, appropriate for use with this method, will be available from the U.S. Environmental Protection Agency, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268.

8.2.2 Using a pipet, add 1.00 mL of the check sample concentrate to each of a minimum of four 1,000-mL aliquots of reagent water. A representative wastewater should be used in place of the reagent water, but one or more additional aliquots must be analyzed to determine background levels, and the spike level must exceed twice the background level for the test to be valid. Analyze the aliquots.

8.3 It is recommended that the laboratory adopt additional quality assurance practices for use with this method. The specific practices that are most productive depend upon the needs of the laboratory and the nature of the samples. Field duplicates may be analyzed to monitor the precision of the sampling technique. Whenever possible, the laboratory should perform analysis of standard reference materials and participate in relevant performance evaluation studies.

9.0 Procedure

9.1 Sample Collection--

9.1.1 Wastewater collection methods--It is recommended that samples be taken which reflect the "normal" state of the sample site. Periodic sampling of the same site over several months may also be valuable in providing information on peak periods of activity. For aqueous samples, the most common sampling procedure is a manual grab collection of the volume needed for analysis. Once collected, samples should be placed in the sealed amber glass bottles and held at 4 C during any storage or shipping. The head space in the container should be reduced by complete filling of the container or by replacement with a N_2 blanket. Table 5 identifies the volume of sample required.

9.1.2 Wastewater sample custody--An example of a sample custody form is shown in Figure 12. This form is from EPA document EPA-600/881-024 (Brusick and Young 1981). Either this EPA form or its equivalent should be prepared at the time the sample is collected, and a copy of the form should accompany the sample through all phases of processing and bioassay.

9.1.3 Storage and use of sample--It is strongly recommended that all samples be processed (separated, extracted and concentrated) within 14 days after collection and completely analyzed within 40 days of concentration and solvent exchange.

9.2 Sample Separation into Component Phases--

9.2.1 Each sample container is stored motionless at 4 C for 24 h after receipt. At this point, a gravity separation of phases will occur. Any nonaqueous liquid phases identified in the liquid component of the sample will be separated from the containers, combined into one sample and processed as a nonaqueous liquid waste.

Any solid sediment on the bottom of the sample container will be collected, combined into a single sample and processed as a waste solid.

The aqueous phase, with or without suspended particles will be recovered and treated in one of three methods (see Section 9.2.3).

9.2.2 Each liquid phase recovered must be weighed to the nearest gram and the volume measured. The solid sediment is weighed (net weight to the nearest gram). Like phases are combined into a common vessel for processing. Once combined, total weights and/or volumes are calculated. Storage conditions are the same as defined for the initial collected sample. No attempts to control

TABLE 5. RECOMMENDED VOLUMES AND STORAGE OF SAMPLES

Component	Volume/Weight	Storage Conditions
Collected Sample	Minimum of 30 L	4 C, dark
Gravity Separation Method	Minimum of 30 L	4 C, dark motionless
Sample for Resin Column Concentration	10 L	4 C, dark glass or Teflon-lined vessel
Liquid/Liquid Extraction Method	3 L	4 C, dark glass or Teflon-lined vessel
Solids for Solid Waste Extraction	500 g net weight	4 C, dark closed container
Extracted/Concentrated/ Solvent Exchanged Sample for Bioassay	10 mL	4 C, amber glass vial with Teflon cap liner

I. SAMPLE INFORMATION

1. Sample No. ____________________ Collection Date ____________
2. Sampling Site[(a)] ____________________________________
3. Field Sampling Manager (on-site) ____________________
4. Contractor ____________________ Contract No. ____________
5. EPA Project Officer ______________ Program Name ____________
6. Source Sampled ____________________________________
7. Discharge Rate of Source (Volume/Time) ____________________
8. Quantity Sampled/Units ____________________________
9. Sample Description (liquid, slurry, solid, extract, appearance, etc.)

____

10. Other Information as Applicable

Collection temp. ______________ Sampling location __________
pH ____________________ Sampling technique __________.
Other ____

II. HANDLING & SHIPPING

1. Describe Sample Treatment Prior to Shipping (e.g., transfers, extractants, stored undiluted, grinding, solvents used) ____________

____

2. Field Storage and Shipping Conditions

Container	Temperature	Light
___ Amber Glass	___ Ambient	___ Shield from light
___ Polyethylene Bottle	___ Refrigerate (0 to 4 C)	
___ Coated Bag or Bottle	___ Freeze (-20 C)	
___ Teflon or Tedlar Bags	___ Dry Ice	
___ Other ____________		

3. Approximate Time in Storage and Time in Shipping ________________
4. Sample Shipped to ________________________________
5. Mode and Carrier for Shipping ______________________
6. Comments ____

____

(This form should be completed by the on-site sampling manager and accompany each sample.)

(a) Graphically illustrate site of collection.

Figure 12. Sample information.
(Extracted from: IERL-RTP Procedures Manual Level 1 Environmental Assessment) Biological Tests EPA-600/8-81-024, October, 1981. pp. 138.)

pH or change the pH of the sample are required. The initial sample containers should be rinsed three times with methylene chloride. The final rinse collection will be concentrated and added to the final extract sample prior to solvent exchange.

9.2.3 The liquid phase recovered from the sample will be processed by one of the following methods:

a. If the sample has <5% suspended solids by weight (reference for method TSS), the sample may be extracted and concentrated by the techniques described in the Drinking Water Protocol, with the addition of ASTM Celite prefilter to the concentrator apparatus.

b. If the sample has >5% suspended solids by weight, the sample may be further separated by high-pressure filtration or high-speed centrifugation into liquid (<5% suspended solids) and solids. These two phases can be processed further by the Drinking Water Protocol and Solid Waste Protocol, respectively.

c. If the sample has >5% suspended solids by weight, it may be processed by a liquid/liquid extraction method (Section 9.3). If the bioassay from the sample recovered from this method is negative, consideration should be given to processing a retained liquid phase (10 L) by the method described in Section 9.2.3.b.

9.3 Separatory Funnel Liquid-Liquid Extraction Method--

9.3.1 Shake the sample, contained in a gallon amber glass bottle, to assure homogeneity. Samples are usually extracted using separatory funnel techniques. If emulsions will prevent achieving acceptable solvent recovery with separatory funnel extractions, continuous extraction may be used.

9.3.2 Measure two 1,500-mL sample aliquots into two 2000-mL separatory funnels. Check the pH of the sample with wide-range pH paper and adjust to pH 11 with 10N sodium hydroxide.

9.3.3 Add 150 mL of methylene chloride to each separatory funnel, and extract the sample by shaking the funnels for two minutes with periodic venting to release excess pressure. Allow the organic layers to separate from the water phases for a minimum of ten minutes If the emulsion interface between layers is more than one-third the volume of the solvent layer, the analyst must employ mechanical techniques to complete the phase separation. The optimum technique depends upon the sample, but may including stirring, filtration of the emulsion through glass wool, centrifugation or other physical methods. Combine the methylene chloride extracts in a 1,000-mL Erlenmeyer flask. If the emulsion cannot be broken (recovery of less than 80% of the methylene chloride, corrected for the water solubility of methylene chloride), transfer the sample, solvent and emulsion into the extraction chamber of a continuous extractor and proceed as described in Section 10.

9.3.4 Add 100 mL of methylene chloride to each separatory funnel, and repeat the extraction procedure a second time, combining all extracts in the 1,000-mL Erlenmeyer flask. Perform another 100-mL extraction in the same manner.

9.3.5 Adjust the pH of the aqueous phases to less than 2 using the sulfuric acid solution. Serially extract the sample with 150, 100 and 100 mL of methylene chloride per separatory funnel. Combine the extracts with previous extracts in the 1,000-mL Erlenmeyer flask. Notice that additional fractionation could be achieved here, if requested, by not combining these extracts (organic acids) with previous extracts (organic base/neutrals) but, rather, processing each separately through the remaining procedure.

9.3.6 Assemble a Kuderna-Danish (K-D) concentrator by attaching a 25-mL concentrator tube to a 500-mL evaporative flask. Other concentration devices or techniques may be used in place of the Kuderna-Danish. Pour one half of the combined extract through a drying column containing about 10 cm of anhydrous sodium sulfate, and collect the extract in the K-D concentrator. Add one or two clean boiling chips and attach a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL of methylene chloride to the top of the column. Place the K-D apparatus on a hot water bath (60 to 65 C) so that the concentrator tube is partially immersed in the hot water, and the entire lower rounded surface of the flask is bathed with hot vapor. Adjust the vertical position of the apparatus and the water temperature as required to complete the concentration in 15 to 20 minutes. At the proper rate of distillation, the balls of the column will actively chatter, but the chambers will not flood with condensed solvent.

9.3.7 When the apparent volume of liquid reaches approximately 10 mL, remove the K-D apparatus from the water bath and add the remaining combined extract through the drying column and into the K-D apparatus. Rinse the Erlenmeyer flask and column with 25 mL of methylene chloride to complete the quantitative transfer. Add two clean boiling chips and continue concentration on the water bath until the liquid reaches 10 mL. Remove the K-D apparatus from the water bath and allow it to drain and cool for at least 10 minutes. Remove the Snyder column and rinse the flask and its lower joint into the concentrator tube with 1 to 2 mL of methylene chloride.

9.3.8 Concentrate the methylene chloride extract to 10 mL using the nitrogen evaporator. Do not allow the bath temperature to exceed 35 C. An aliquot should be removed for total organic carbon (TOC) analysis (Lentzen et al. 1978). If requested, at this point a 1-mL aliquot may be removed for analysis by gas chromatograph/mass spectrometer using Method 625 (44 FR 233, December 3, 1979). Transfer the extract to a tared, 3-dram glass vial that is etched at the 5-mL mark. Weigh the vial and its contents and record the weight of the residue. Dilute the sample extract to the 10-mL mark with dimethyl sulfoxide and attach the Teflon-lined screw cap to the vial. From the TOC determination, the DMSO extract should be recorded as mg organics/mL DMSO. Refrigerate the extract until ready for Ames testing.

9.3.9 If no aliquot was removed for analysis by gas chromatograph/mass spectrometer, the DMSO extract represents a concentration factor of approximately 600x. If an aliquot was removed for analysis by gas chromatograph/mass spectrometer, the DMSO extract represents a concentration factor of approximately 540x.

10.0 Calculations

All measurements of mutagenic activity should be expressed as revertants per liter of emission and as a rate (revertants/liter/hour). In order to generate these values, data for revertants/mg organics extracted by each sample phase depicted in Figure 11 are needed.

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SECTION 5

NONAQUEOUS LIQUID WASTES

REVIEW OF THE LITERATURE

Introduction

Nonaqueous liquid environmental samples such as coal or petroleum products and process effluents have been shown to contain mutagenic and cancer causing agents (Heuper 1953, Gradiski et al. 1983, Schultz et al. 1983). Several short-term bioassays have been used to characterize the adverse effects of these environmental mixtures (Epler et al. 1979). To make maximum use of these bioassays, the samples must be prepared in such a way that they will be compatible with the assaying system, and yet be representative of realistic exposure impact.

Efforts to substantiate the hazard of nonaqueous liquid waste and industrial byproducts have been hampered due to the complexity of the problem. The difficulties lie in the nature of analysis, in that (a) a nonaqueous sample is to be tested in an aqueous system, (b) inhibitory and/or synergistic effects of the complex mixtures and toxicity of one or more particular components can complicate the resulting interpretation and (c) limitations exist for each test for detecting all types of carcinogens; for example, certain components in the unknown sample can be negative in one system and mutagenic in another system with different metabolic activation capabilities or with different mutational targets.

Nonaqueous liquid environmental samples are those containing less than 5% solids. The sample can contain mixtures of volatile (b.p. 36 C to 100 C), moderately volatile (b.p. 100 C to 300 C) and nonvolatile (b.p. $\geq$300 C) organic compounds. In addition, the samples are generally entities of poorly defined and possibly continuously changing chemical composition.

The method for sample preparation can be a simple one, such as evaporation to concentrate the sample, or a complicated, detailed fractionation scheme. The technique used most successfully to date is to separate the constituents of the mixture into a manageable number of chemically distinct fractions. Each fraction is then bioassayed to determine whether mutagenic constituents of any type are present independent of synergism, toxic interferences or other complications as a result of the interaction of constituents of widely differing properties (Guerin et al. 1978, 1982). The methods of separation also take cognizance of the chemical nature of the compounds to be separated and, more specifically, of differences in physical properties (boiling point, solubility, etc.), polarity and acidic/basic characteristics.

However, the primary requirement is that all of the constituents present in the starting material be recovered in the fractions to be bioassayed.

The methods for preparation of complex mixtures for in vitro bioassays must meet requirements that are different from those designed for chemical characterization. For bioassay purposes, it is important to produce a material that is suitable for biotesting and whose composition is equivalent to that in the original sample. In addition, the sample preparation procedures should be simple and cost-effective.

Several methods have been developed to prepare nonaqueous liquid environmental samples for in vitro mutagenicity assays, especially the Ames Salmonella test (Ames et al. 1975), which is one of the most widely used methods for assessing the adverse biological properties of environmental complex mixtures (Ames 1979). Samples have been introduced into the test medium as organic solvent extracts of the crude sample, as surfactant-stabilized organic extracts and as a series of chemical class fractions of the starting material. The available literature on this subject is reviewed and a protocol for the preparation of nonaqueous liquids for evaluation in the Ames Salmonella assay is established.

Summary of Currently Available Methods

The available literature on the preparation of nonaqueous liquids for short-term bioassay has focused primarily on samples derived from the petroleum and synfuel industries. The ultimate objective of those reports was to simplify complex matrices by fractionation to the point where chemical identification of active mutagens becomes more realistic. Although our primary objective is to evaluate the mutagenic potential of nonaqueous liquids, the literature pertaining to chemical identification is quite relevant to the preparation of fractions for mutagenicity screening.

Guerin et al. (1980) evaluated the mutagenic potential of petroleum substitute using a chemical class fractionation coupled with the standard Ames test. A liquid sample was evaporated at 40 C under water aspiration, and the remaining oil was fractionated by acid-base extractions into acid, base and neutral fractions. The procedure first subjected the sample to partitioning between ether and 1N HCl. Alkaline constituents of the sample concentrated in the acidic aqueous phase, whereas acid and neutral constituents concentrated in the organic phase. Subsequent mixing of the organic phase with 1N NaOH preferentially extracted the acidic constituents. The constituents of the aqueous phase were back-extracted into ether following pH adjustment. Four fractions (acids, bases, neutrals and any insoluble residue formed) thus resulted for biological testing. Further fractionation was achieved by Rubin et al. (1976) and Jones et al. (1977) using alumina and Sephadex LH-20 column chromatography. Lipophilic and hydrophilic constituents were separated by hexane and methanol. Further fractionation of the lipophilic components was achieved by using tetrahydrofuran and isopropanol. The gel chromatographic procedure thus produced the following fractions: hydrophilic, polymeric, hydrogen bonding, aliphatic, simple aromatic and polyaromatic.

Chemical class fractionation similar to that used by Rubin et al. (1976) has been reported by Epler et al. (1978, 1979), Pelroy and Petersen (1979), Ho et al. (1980, 1981), Timourian et al. (1982) and Toste and Sklarew (1982). Schoeny et al. (1981) applied a simpler scheme of separation by serial extraction with organic solvents of increasing polarity to products from coal gasification and liquefaction. However, the nonmutagenic samples did not yield mutagenic components upon the fractionation. Using simple silica gel column chromatography and a liquid-liquid extraction method, Matsuoka et al. (1982) demonstrated that the clastogenic effects of the heavy oil may be due to the presence of nitrogen-containing polycyclic hydrocarbons such as azaarenes.

Ma et al. (1983) prepared fossil-derived oils by first dissolving the sample in cyclohexane, followed by extraction with dimethylsulfoxide (DMSO). The solvent was removed from the separated cyclohexane fraction by evaporation under vacuum at 45 C with a rotary evaporator. The DMSO-extractable material was then bioassayed. This procedure partitions lipophilic hydrocarbons into the cyclohexane phase, while the more hydrophilic components, including the polynuclear aromatic hydrocarbons, partition into DMSO. Thus, the incompatibility of the lipophilic sample with the hydrophilic test medium is alleviated.

Ma et al. (1983) also prepared a DMSO slurry of DMSO-insoluble oils using an ultrasonic bath at ambient temperature to form an emulsion. This procedure yielded about a 33% increase in mutagenicity (revertants/mg) relative to the DMSO-cyclohexane extraction procedure cited above. Surfactant Tween 80(a) water emulsions of oil samples also showed lower activity than the DMSO slurry. However, with the slurry or emulsion technique, uniformity of the sample must be maintained to obtain reproducible results.

Hermann et al. (1980) used nitromethane or DMSO to extract oil samples dissolved in cyclohexane. The nitromethane or DMSO was vacuum-distilled at 70 C, and the residue was dissolved in DMSO for Ames testing. For the detection of mutagenic activity in oil resulting from benzo(a)pyrene (BP), the extraction procedure was slightly better with DMSO than with nitromethane. These investigators also compared the above solvent extraction procedure with the testing of solubilized oils in aqueous phospholipid or Tween 80(a) suspensions. The extraction of BP from oil by these solubilizing agents gave results that were comparable with those obtained with pure BP in DMSO. These findings suggest that the full mutagenic potential of the suspended oil can be achieved by the detergent technique, thus eliminating the concern as to extraction efficiency.

Nonaqueous liquids may contain both volatile and nonvolatile compounds. If the volatile components are mutagens that can be detected in the Salmonella desiccator assay (Baden et al. 1977, Simmons et al. 1977), quantitative analysis of moderately volatile materials can be achieved by a gas chromatographic procedure called total chromatographable organics (TCO) analysis (Lentzen et al. 1978, Section 9.4.1). However, TCO analysis is not required in terms of sample preparation for the Ames bioassay. If the nonvolatile organic components in the sample are mutagens detected either in the plate incorporation assay or in the preincubation assay, a gravimetric (GRAV) analysis is performed (Lentzen et al. 1978, Section 9.4.2). Briefly, by this simple procedure, an aliquot of the sample is evaporated in a preweighed aluminum weighing pan and then dried to a constant weight. The residue weight is determined, and the GRAV nonvolatile organic content is calculated from the residue weight and reported as one number for the whole sample. At least 10 mg of sample residue should be weighed, but no more than 10% of the sample should be used for GRAV analysis. Highly viscous liquids and pastes are weighed directly, placed in a suitable solvent and dosed on a weight-per-volume basis.

(a) Registered trademark.

From the above literature reports, we conclude that the best way to evaluate the initial mutagenicity of nonaqueous liquids is to test them "neat" or as DMSO suspensions or solutions. If no mutagenic activity is found, acid, base and neutral partitioning should be performed to fully evaluate the mutagenic potential of the sample.

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NONAQUEOUS LIQUID WASTES WORKGROUP REPORT

Workgroup Tasks

Summary of Tasks--

In summary, nine tasks were identified during the general sessions and workgroup discussion. These nine tasks are listed under the Summary of Workgroup Progress.

Key Peer Review Comments--

There were no unresolved written peer review comments from the pre-meeting review.

Summary of Workgroup Progress--

a. Definition - Nonaqueous liquid wastes are those liquids whose major component is not water, including water-soluble organic liquids such as acetone from an industrial process, water-insoluble liquids such as dichloromethane from a similar industrial process and liquids that might be composed of light to heavy oils.

b. Storage - The samples, regardless of source, should be stored in Teflon-capped amber-glass bottles with zero headspace or argon blanket, whichever is applicable, at 4 C or lower. Aliquots, such as 10 mL per vial, should be prepared before storage and transportation. The neat material should be tested as soon as possible.

c. Water-soluble compounds - This is a non-issue because we will use organic solvents for extraction, and these compounds will be extracted.

d. Solvent - We recommend the use of dichloromethane because it is a universal solvent and has been applied to most other media types and because it is easy to obtain high purity dichloromethane.

   Note: We recommend that a research project be done to compare CH_2Cl_2 and diethyl either for extraction efficiency.

e. pH - We will test the neat sample as is, without adjustment of pH. For fractionation, we suggest extracting bases with 1N HCl first, and then separating the acids and neutral fraction.

f. Blank - (1) For the Ames test, solvent control and positive controls are necessary for all three assays to be performed.

(2) A solvent blank should be done using the fractionation scheme. The residues should be subjected to the Ames test.

g. Internal standards - We suggest spiking half of the sample with known mutagens. Some possible standards could be benzo(a)pyrene for neutral mutagens, 2-aminoanthracene for base mutagens and p-nitrobenzoic acid for acid mutagens.

h. Recovery - The weight of each fraction and the original sample should be recorded, and the recovery rate should be calculated. The fractions should be reconstituted and assayed together with each fraction to evaluate additive/synergistic/inhibitory effects.

i. Vapor phase organics and modification of the Ames test - We will use the desiccator assay to detect the vapor phase organic mutagens.

Summary of Key Workgroup Discussions

Major Consensus Opinions--

The consensus of opinions is that the proposed protocol is sound. Specific details that were not delineated in the original protocol have been agreed upon.

Unresolvable Issues--

There are no unresolved issues.

Evaluation of Proposed Protocol

Adequacy--

The protocol is believed to be adequate for the majority of nonaqueous liquids.

Validity--

The protocol should be validated in a range of water-soluble and water-insoluble organic matrices.

Limitations--

a. There is the potential for alteration of the mutagenic activity using an acid-base extraction fractionation procedure.

b. If toxicity is observed in the DMSO-acids, bases or neutrals fractions, we are not recommending further fractionation.

c. Low concentrations of highly polar mutagens may not be detected as a result of the fractionation procedures.

Comparison to the Other Five Media Protocols--

The fractionation procedure should be identical to those in the soil and solid wastes protocols if HPLC methods are not adopted.

Other Data or Information Requirements

Information Gaps--

Data are needed for the selection of an optimum extraction solvent and sample storage condition.

Research Program Needs--

(a) No data on other liquids besides oils. (b) Chromatographic methods for sample fractionation should be studied.

PROTOCOL FOR THE PREPARATION OF NONAQUEOUS LIQUID WASTES FOR MUTAGENICITY TESTING

1.0 Scope and Application

1.1 This method covers the preparation of nonaqueous liquid environmental samples for incorporation into the Ames mutagenicity assay (Ames et al. 1975, Maron and Ames 1983).

Nonaqueous liquids can range from very simple, one-component systems (such as an organic solvent) to highly complex chemical mixtures such as crude oils or industrial wastes. In many cases, the neat material can be tested directly in an *in vitro* system without compromising the nature of the starting material. In other cases, substantial handling of the sample may be required to fully evaluate the mutagenic potential of the liquid. In the latter case, it is important that the integrity of the sample be maintained and that the material undergoing bioassay reflect the material that was sampled.

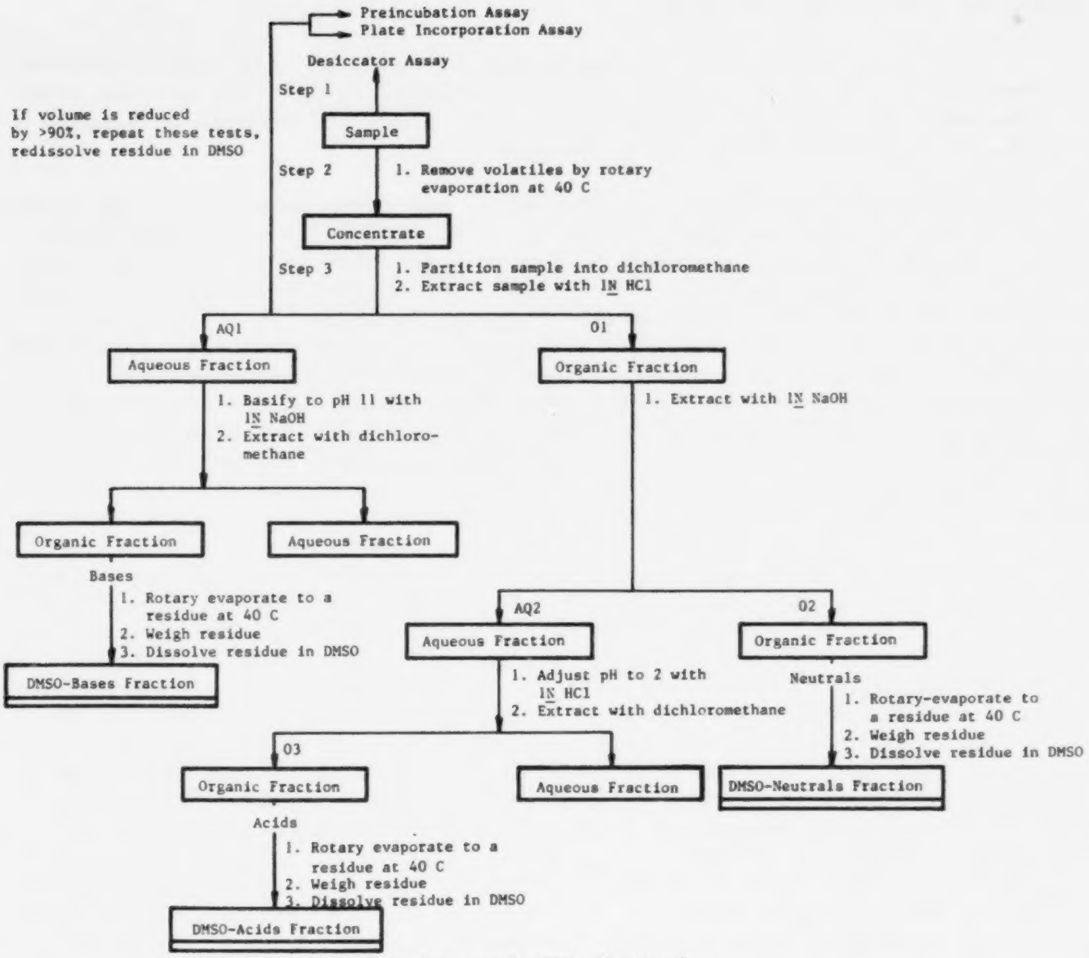
1.2 This method is applicable to volatile mutagenic compounds which can be detected by the desiccator assay (Baden et al. 1977, Simmons et al. 1977), a modified method of the Ames *Salmonella*/mammalian-enzyme assay. The method is also applicable to organic compounds that can be partitioned or emulsified into dimethylsulfoxide (DMSO) and are stable to acid base solvent partitioning. However, the user is cautioned that not all chemicals are stable through this procedure. For example, benzidine can be subject to oxidation losses during the solvent concentration; α-BHC, δ-BHC, endosulfan I and II and endrin are subject to decomposition under the alkaline conditions of the extraction step and hexachlorocyclopentadiene and N-nitrosodiphenylamine decompose at higher temperatures (EPA 1982, 1984).

1.3 This method is restricted to use by or under the close supervision of analysts experienced in traditional organic extraction procedures and good laboratory practices. Each analyst must demonstrate the ability to generate acceptable results with this method.

1.4 The neat materials and the final extracts are subjected to the Ames mutagenicity assay using the procedures of Williams and Preston (1983).

2.0 Summary of Method

To determine the mutagenic potential of nonaqueous liquids as measured by the Ames *Salmonella*/mammalian-enzyme assay, the following protocol is recommended (also diagrammed in Figure 13) for the sample preparation. In Step 1, the desiccator assay is performed on the neat material. The desiccator assay allows the detection of volatile mutagens (such as chlorinated solvents) that are often missed in the plate incorporation and preincubation assays


Preincubation Assay
Plate Incorporation Assay
Desiccator Assay
Step 1
If volume is reduced by >90%, repeat these tests, redissolve residue in DMSO
Sample
Step 2
1. Remove volatiles by rotary evaporation at 40 C
Concentrate
Step 3
1. Partition sample into dichloromethane
2. Extract sample with 1N HCl
AQ1
Aqueous Fraction
01
Organic Fraction
1. Basify to pH 11 with 1N NaOH
2. Extract with dichloromethane
1. Extract with 1N NaOH
Organic Fraction
Aqueous Fraction
Bases
1. Rotary evaporate to a residue at 40 C
2. Weigh residue
3. Dissolve residue in DMSO
DMSO-Bases Fraction
AQ2
Aqueous Fraction
02
Organic Fraction
1. Adjust pH to 2 with 1N HCl
2. Extract with dichloromethane
Neutrals
1. Rotary-evaporate to a residue at 40 C
2. Weigh residue
3. Dissolve residue in DMSO
03
Organic Fraction
Aqueous Fraction
DMSO-Neutrals Fraction
Acids
1. Rotary evaporate to a residue at 40 C
2. Weigh residue
3. Dissolve residue in DMSO
DMSO-Acids Fraction

Each DMSO fraction from Step 3 will be bioassayed.

Figure 13. Sample preparation scheme for bioassay.

(Baden et al. 1977, Simmons et al. 1977). In addition, a suspension of the neat material (20 mg/mL) is prepared by ultrasonication (5 min at room temperature) in high purity DMSO (Selby et al. 1983, Wang et al. 1978) and tested in the plate incorporation and the preincubation assays. The preincubation assay allows the detection of certain mutagens, such as dimethylnitrosamine, that require additional time for activation by the mammalian enzymes (Haworth et al. 1983). A positive response in any of the above three assays indicates the presence of mutagenic components, and the evaluation process is completed.

If a toxic response or a combination of toxic and negative responses are observed in all three assays, the neat material is diluted, and the assay is repeated. The dilution is made in 10, 100, 1,000 and greater fold until a negative or a positive response is observed.

If a negative response is observed in all three assays, in Step 2, the liquid is concentrated by rotary evaporation at 40 C under water aspiration. These conditions are gentle enough to remove most volatile solvents (acetone, methanol, etc.) and to maintain the integrity of thermally labile components. If the volume or weight can be reduced by a factor of at least ten by the evaporation procedure, the residue is retested in the plate incorporation and preincubation assays. If a solid residue is produced, it is dissolved in a minimum amount of dimethylsulfoxide (DMSO) prior to assays. If the liquid is not effectively concentrated by the evaporation procedure or produces a negative response, it is dissolved in an organic solvent and partitioned into acidic, basic and neutral fractions. If there is any standardization to be applied, all samples should be handled identically.

In Step 3, the liquid is dissolved in dichloromethane and extracted with 1N HCl. The aqueous layer (AQ_1) is adjusted to pH 11 with 1N NaOH and extracted with dichloromethane to yield the basic fraction. This fraction is dried over anhydrous sodium sulfate, filtered and rotary-evaporated at 40 C to an oil or residue. The residue is weighed and dissolved in a minimum of DMSO and is called the DMSO-bases fraction. The organic layer (O_1) from the acid extraction is extracted with 1N NaOH to produce aqueous (AQ_2) and organic (O_2) fractions. The AQ_2 fraction is adjusted to pH 2 with 1N HCl and extracted with dichloromethane (O_3). The O_3 fraction is dried over anhydrous sodium sulfate, filtered and evaporated to a residue. The residue is weighed and dissolved in a minimum of DMSO and is labeled DMSO-acids. The O_2 fraction is handled in a fashion similar to that for the O_3 fraction and is worked up to become the DMSO-neutrals fraction. All extractions are performed three times with equal volumes of base or acid. The fractionation scheme is shown in Figure 13. The DMSO-acids, DMSO-bases and DMSO-neutrals fractions are then evaluated for mutagenicity in the plate incorporation and preincubation assays.

The fractionation method is preferred because it isolates components that are toxic or that might otherwise interfere with the assay. Thus, the potential to find all the contributors to mutagenicity is increased. Also, the distribution of activity in the various fractions provides a preliminary estimate of the types of chemicals responsible for the mutagenicity. Furthermore, the potential for synergistic or antagonistic effects associated with the crude material is greatly reduced.

3.0 Definitions

Nonaqueous liquid waste environmental samples are those liquids whose major component is not water, including water-soluble organic liquids (such as acetone), water-insoluble liquids (such as dichloromethane) or liquids that might be composed of light to heavy oils (such as coal or petroleum products or process effluents). The samples contain less than 5% solids and may contain mixtures of volatile (b.p. 36 C to 100 C), moderately volatile (b.p. 100 C to 300 C) and nonvolatile (b.p. 300 C) organic compounds. In addition, the samples are generally entities of poorly defined and possibly continuously changing chemical composition.

4.0 Interferences

4.1 Method interferences may be caused by contaminants in solvents, reagents, glassware and other sample processing hardware that lead to discrete artifacts. All of these materials must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks.

4.1.1 Glassware must be scrupulously cleaned. All glassware should be cleaned as soon as possible after use by rinsing with the last solvent used in it. This should be followed by detergent washing with hot water and rinses with tap water and reagent water. It should then be drained dry and heated in a muffle furnace at 400 C for 15 to 30 min. Some thermally stable materials, such as PCBs, may not be eliminated by this treatment. Solvent rinses with acetone and pesticide-quality dichloromethane may be substituted for the muffle furnace heating. Volumetric glassware should not be heated in a muffle furnace. After drying and cooling, glassware should be sealed and stored in a clean environment to prevent any accumulation of dust or other contaminants. It should be stored inverted or capped with aluminum foil.

4.1.2 The use of high purity reagents and solvents helps to minimize interference problems. Purification of solvents by distillation in all-glass systems may be required.

4.2 Matrix interferences may be caused by contaminants that are coextracted from the sample. The extent of matrix interferences will vary considerably from source to source, depending upon the nature and diversity of the system being sampled.

5.0 Safety

5.1 The toxicity or carcinogenicity of each reagent used in this method has not been precisely defined. However, each chemical compound should be treated as a potential health hazard. From this viewpoint, exposure to these chemicals must be reduced to the lowest possible level by whatever means available. The laboratory is responsible for maintaining a current awareness file of OSHA regulations regarding the safe handling of the chemicals specified in this method. A reference file of material data handling sheets should also be made available to all personnel involved in the chemical and mutagenicity analyses.

5.2 An example of safety procedures is described in detail as follows:

All substances to be tested, as well as the positive controls, will be handled as if they were carcinogens. Upon receipt, the container(s) holding the test articles is taken directly to the chemical repository, where the shipping containers are inspected for damage and for agreement with the packing invoice. The test articles are then removed from the shipping container(s) and placed in secondary containers, which are stored according to the instructions in the safety and handling documents, if available.

The chemical repository is a limited-access area with entry restricted to one technician and two back-up individuals. All personnel are required to sign in and out on a log sheet and wear protective clothing (two pairs of dissimilar disposable gloves, Tyvek full-body suits and shoe covers), which is disposed of properly after use. The room contains a glove box for handling test articles, a ventilated and enclosed Mettler balance, and appropriate ventilated storage cabinets under negative pressure to house the test articles at room temperature, 4 C, or -10 C.

Aliquots of the test article are weighed in screw-top, glass test tubes and transferred to the pass-through window in a secondary container. An inventory is kept of all articles entering and leaving the repository. Available in the repository, or adjacent to it, are a half-mask chemical cartridge respirator, a voice-activated intercom, a fire extinguisher, a safety shower and an eye wash. The repository is equipped with emergency lighting in the event of a power failure.

All entrances into the regulated areas are posted with the appropriate warning signs stating that a hazardous chemical is present and that eating, drinking or smoking in these areas is prohibited. Also, emergency procedures and telephone numbers to be called in case of an emergency are posted on the doors.

Plastic and paper waste are disposed of in a plastic-lined, cardboard waste container. Glass waste (pipets, culture tubes, dilution tubes) is placed in plastic-lined, 30-gal fiber drums. Liquid waste is collected in 1-gal plastic, screw-capped containers. All waste containers are vented and double-bagged before being sealed with duct tape, labeled as chemical carcinogens and disposed of regularly by health and safety specialists.

Entry into the designated limited-access areas for the proposed research requires an appropriate medical evaluation, with additional evaluations at yearly intervals or after possible accidental exposure. All personnel working within these regulated areas must sign in/out on a log sheet posted at the entrance and wear protective clothing (two pairs of dissimilar disposable gloves, Tyvek disposable gowns with a solid front and shoe covers). Maintenance workers and other individuals requiring temporary access wear disposable paper coveralls, shoe covers and gloves. Protective clothing is discarded after use. All personnel wash their hands before leaving the regulated areas.

Work with a test article is performed on disposable, plastic-backed absorbent pads within Baker Class II, Type B hoods (NCB-4 or NCB-6). The exhaust air is filtered by a HEPA filter in the hood and by charcoal filters on the roof. Hood surfaces are wiped with ethanol and disinfectant before and after completion of an experiment. The hoods are inspected and certified once a year by an outside testing agency. The charcoal filters are tested for effectiveness every week by laboratory personnel. All hoods are equipped with warning devices to indicate insufficient air flow during a power failure or because of a mechanical or other failure.

Laboratory personnel retrieve the secondary container with the requested amount of test article from the pass-though window of the chemical repository. All further manipulations of the test article are conducted in the hoods. Any chemical solutions remaining after completion of the test are disposed of in properly labeled 1-gal plastic containers.

Spill packs containing absorbent material and other items for cleaning up minor spills are available in the regulated areas. All laboratories are equipped with fire extinguishers, first-aid kits, hand-held eye washers and telephones. Emergency numbers to be called and signs outlining the correct procedures to follow in case of an emergency are posted in all laboratories.

The laboratory should comply with the applicable state and federal Occupational Safety and Health Administration regulations and with federal security and safety requirements specified under the Williams-Steiger Occupational Safety and Health Act of 1970. The laboratory should also follow the National Cancer Institute Safety Standards for Research Involving Chemical Carcinogens, DHHS Publication No. NIH-76900.

6.0 Apparatus and Equipment

All specifications and brand names are suggestions only.

6.1 All the equipment and materials required for the Ames bioassay (Williams and Preston 1983).

6.2 Desiccator - 9 L, all glass, with lid and porcelain platform.

6.3 Glass petri dishes - 15 x 100 mm.

6.4 Disposable glass pipette - 1, 5 and 10 mL.

6.5 Graduated cylinder - 100 or 250 mL.

6.6 Vials - Amber glass, with Teflon-lined screw caps.

6.7 Sample bottles - 1,000-mL to 5,000-mL glass with Teflon-lined screw caps.

6.8 Beakers - 100 and 250 mL.

6.9 Flasks - Erlenmeyer, 250 mL.

6.10 Separatory funnels - 250 mL with Teflon stopcocks.

6.11 Rotary evaporator - Buchi or equivalent.

6.11.1 Flasks - Round bottom, 50, 125, 250 and 500 mL.

6.11.2 Traps - Glass.

6.11.3 Clamps.

6.11.4 Water bath - Heated with temperature control (±2 C).

6.12 Balance - Analytical, capable of accurately weighing 0.0001 g.

6.13 Nitrogen evaporator - Equipped with Teflon or glass jets and temperature control, Meyer N-evap Model 112 or equivalent.

6.14 Ultrasonic Bath - Bransonic 12 or equivalent.

7.0 Reagents and Consumable Materials

7.1 Acetone - "Distilled in Glass" or equivalent, stored in original containers and used as received.

7.2 Dichloromethane - "Distilled in Glass" or equivalent, stored in original containers and used as received.

7.3 Dimethysulfoxide - High purity, supplied by Pierce or equivalent, stored in original containers and used as received. Should be stored under nitrogen or argon when opened.

7.4 Reagent water - Reagent water is defined as a water in which an interference is not observed at the method detection limit of each parameter of interest.

7.5 Hydrochloric acid solution (1N) - Slowly add 8.3 mL of HCl (12N) to reagent water and dilute to 100 mL.

7.6 Sodium hydroxide solution (1N) - Dissolve 4 g NaOH in reagent water and dilute to 100 mL.

7.7 Sodium sulfate (Na_2SO_4) - Anhydrous, granular. Clean by overnight Soxhlet extraction with dichloromethane, drying in an oven at 110 to 160 C and then heating to 650 C for 2 h. Store in a glass jar tightly sealed with Teflon-lined screw cap.

7.8 Nitrogen gas - Cylinder, secured and grounded.

7.9 Argon gas - Cylinder, secured and grounded.

8.0 Sample Collection, Preservation and Handling

8.1 Grab samples must be collected in glass containers. The containers should be prewashed with high purity acetone and dried before use. A sample collection form, as illustrated in Figure 14, should be reported on each sample. Aliquots, such as 10 mL per vial, should be prepared before storage and transportation. The samples, regardless of source, must be stored in Teflon-capped amber glass bottles with zero headspace or under a blanket of inert gas (e.g., argon). The samples must be iced or refrigerated at 4 C or below from the time of collection until preparation.

8.2 All sample preparation and testing procedures must be performed under yellow/gold lighting or equivalent to avoid photoactivation or decomposition of the product.

8.3 Samples can be warmed to room temperature over a 1- to 2-h period prior to testing. If the samples are to be reused, they should be purged with argon gas, sealed and stored at 4 C or below.

8.4 All samples should be tested and processed as soon as possible.

9.0 Calibration and Quality Control

9.1 Each laboratory that uses this method is required to operate a formal quality control program. The minimum requirements of this program consist of an initial demonstration of laboratory capability and the analysis of spiked samples as a continuing check on performance. The laboratory is required to maintain performance records to define the quality of data that is generated. Ongoing performance checks must be compared with established performance criteria to determine if the results of analyses are within accuracy and precision limits expected of the method.

9.2 To establish the ability to generate acceptable accuracy and precision, the analyst must perform the following operations with spiked and solvent blank samples.

9.2.1 Surrogate spikes - The laboratory is required to spike all of their samples with the surrogate standard spiking solution to monitor spike recoveries. Suggested surrogate compounds are 2-aminoanthracene, benzo(a)pyrene and p-nitrobenzoic acid. If the recovery for any surrogate standard does not fall within the control limits for method performance, the results reported for that sample must be qualified with respect to the total mutagenicity recovered.

The concentration of the surrogate mutagen should exhibit a response that is at least five times the background spontaneous mutation rate at the highest dose in the dose-response curve. The spiked sample dose-response curve is then compared to the standard curve for total mutagenicity recovered.

9.2.2 Method blank - Before processing any samples, the analyst should demonstrate, through the analysis of an 80-mL aliquot of reagent water, that all glassware and reagents are interference free. Each time a set of samples is extracted or there is a change in reagents, a laboratory reagent blank should be processed as a safeguard against laboratory contamination.

I. SAMPLE INFORMATION

1. Sample No. ____________ Collection Date ____________
2. Sampling Site ____________
3. Field Sampling Manager (on-site) ____________
4. Contractor ____________ Contract No. ____________
5. EPA Project Officer ____________ Program Name ____________
6. Source Sampled ____________
7. Quantity Sampled/Units ____________
8. Sample Description (liquid, slurry, solid, extract, appearance, etc)
____
9. Other Information as Applicable

Collection temp. ____________ Sampling location ____________
pH ____________ Sampling technique ____________
Other ____________

II. HANDLING & SHIPPING

1. Describe Sample Treatment Prior to Shipping (e.g., transfers, extractants, stored undiluted, grinding, solvents used) ____________
____
2. Field Storage and Shipping Conditions

Container	Temperature	Light
____ Amber Glass	____ Ambient	____ Shield from light
____ Teflon bags	____ Refrigerate (0 to 4 C)	
____ Other	____ Freeze (-20 C)	
	____ Dry Ice	

3. Approximate Time in Storage and Time in Shipping ____________
4. Sample Shipped to ____________
5. Mode and Carrier for Shipping ____________
6. Comments ____________
____

(This form should be completed by the on-site sampling manager and accompany each sample. A copy should be forwarded to EPA Project Officer and should be attached to the Final Report).

Figure 14. Sample collection form.
(Extracted from: IERL-RTP Procedures Manual: Level 1 Environmental Assessment Biological Tests EPA-600/8-81-024, October, 1981, 138 pp.)

10.0 Procedure

10.1 Sample Preparation Procedure--

Step 1.A Desiccator Assay (Figure 15)

1. Pour an aliquot of the nonaqueous liquid into a standard glass petri dish to cover the bottom of the dish.
2. Test liquids for mutagenicity following the Desiccator Assay Protocol as described in Section 10.2.3.

Step 1.B Plate Incorporation and Preincubation Assay

1. Dissolve or suspend the test liquid in DMSO at a concentration of 20 mg/mL. For suspending the liquid, use an ultrasonic bath at room temperature for 5 min.
2. Test the solution or suspension using the plate incorporation and preincubation protocols as described in Interim Procedures for Conducting the Salmonella/Microsomal Mutagenicity Assay (Ames Test) (EPA Report-600/4-82-068, Williams and Preston, 1983).
3. If toxicity is observed, perform 10-, 100- and 1,000-fold dilutions of the neat liquid in DMSO until a dose-response or background (negative) response is achieved.

Step 2. Concentration

1. If the test liquid possesses a low viscosity, weigh an aliquot (∿100 g) in a tared round-bottom flask.
2. Concentrate the liquid by rotary evaporation at 40 C, using a Buchi rotary evaporator or equivalent with water aspiration or equivalent.
3. Weigh the residue. If a 10-fold concentration factor is achieved, retest the residue in the plate incorporation and preincubation assays after dissolving or suspending the residue in a minimum amount of DMSO.

Step 3. Fractionation

If a toxic or negative response is observed in Steps 1 or 2, the test liquid is fractionated by acid-base partitioning as described below.

1. Weigh approximately 5 g of the test liquid into a 250-mL Erlemmeyer flask and add 100 mL of high purity dichloromethane.
2. Swirl the flask until the liquid dissolves. If solution is not complete after 10 min, add a magnetic stir-bar and stir the solution on a stir-plate for 1 h. If solution is not complete,

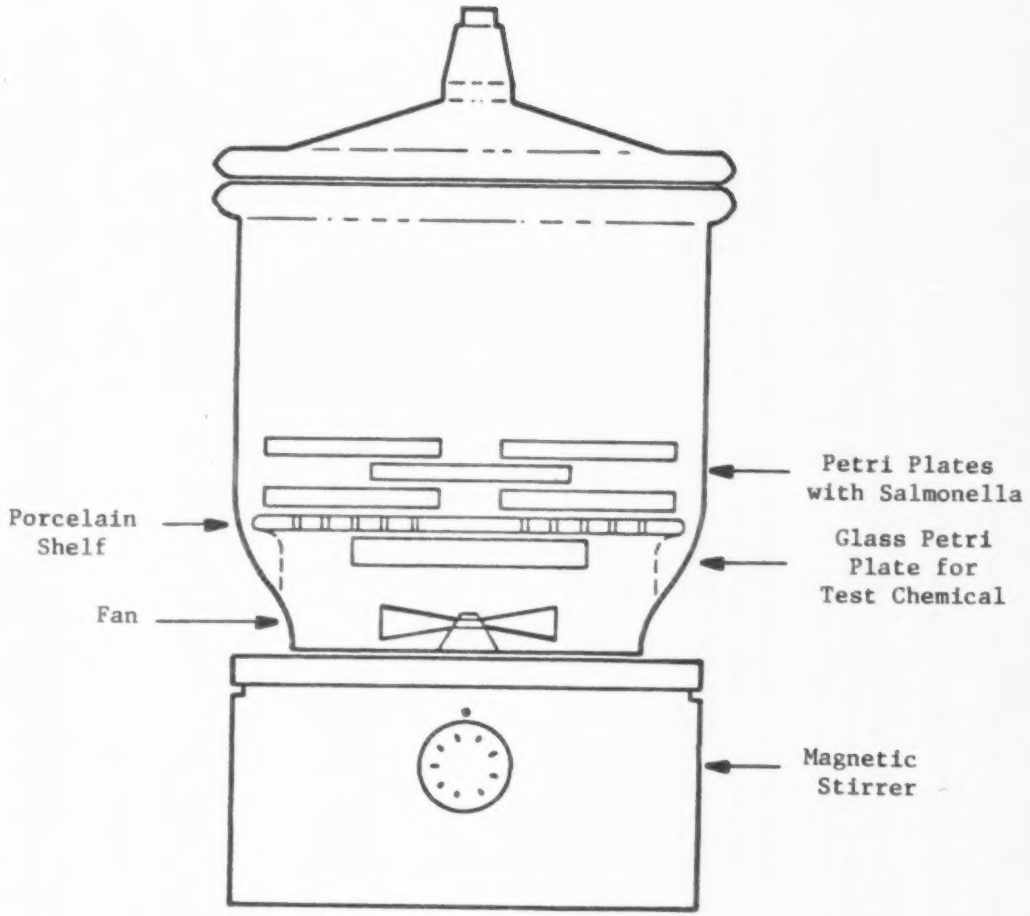

Petri Plates
with Salmonella
Porcelain
Shelf
Glass Petri
Plate for
Test Chemical
Fan
Magnetic
Stirrer

Figure 15. Desiccator assay.

separate the layers using a separatory funnel, and retest the insoluble liquid as described in Step 1 plate incorporation and preincubation assays.

3. The dichloromethane phase is transferred to a 250-mL separatory funnel and extracted with 3 x 75 mL portions of 1N HCl. Save the aqueous (Step 3.4) and organic phases (Step 3.6).

4. The aqueous HCl phase is basified with 1N NaOH to pH 11 and extracted with 3 x 50 mL portions of dichloromethane. The dichloromethane extracts are combined, dried over anhydrous sodium sulfate, filtered and rotary evaporated at 40 C to approximately 4 mL. The dichloromethane concentrate is transferred to a 8-dram glass vial with a Teflon-lined cap with a minimum amount of fresh dichloromethane and blown dry with a gentle stream of nitrogen gas.

5. The residue is capped, weighed and dissolved in a minimum amount of DMSO. The vial is labeled with the sample identification, the concentration and "contains the DMSO-bases."

6. The dichloromethane phase from Step 3 is extracted with 3 x 75 mL portions of 1N NaOH in a 250-mL separatory funnel. Save the aqueous and dichloromethane phases.

7. The dichloromethane phase is dried over anhydrous sodium sulfate, filtered and concentrated to approximately 4 mL by rotary evaporation. The concentrate is transferred to a tared 8-dram vial with a minimum amount of fresh dichloromethane and blown dry with a gentle stream of nitrogen gas.

8. The residue is capped, weighed and dissolved in the minimum amount of DMSO. The vial is labeled with the sample identification, the concentration and "contains the DMSO-neutrals."

9. The aqueous phase from Step 6 is carefully acidified with 1N HCl to pH 2 in a 500-mL separatory funnel. The solution is then extracted with 3 x 100 mL portions of dichloromethane.

10. The dichloromethane extracts are combined, dried over anhydrous sodium sulfate, filtered and concentrated to approximately 4 mL. The concentrate is transferred to a tared 8-dram glass vial and blown dry with a gentle stream of nitrogen gas.

11. The residue is capped, weighed and dissolved in a minimum amount of DMSO. The vial is labeled with the sample identification, the concentration and "contains the DMSO-acids."

12. From the weight of the residues and the initial sample weight, the percent distribution of weight into the DMSO-bases, DMSO-neutrals and DMSO-acids fractions is calculated. Also, the percent

recovery of total mass is determined as the sum of the acid, base and neutral fraction weight divided by the initial sample weight.

13. The DMSO-bases, -neutrals, and -acids are tested for mutagenicity as described in Step 1B.

10.2 The Ames Bioassay Procedure--

The specific test method of the Ames bioassay has been described in detail (Ames et al. 1975, Maron and Ames 1983, Williams and Preston 1983).

10.2.1 Plate Incorporation Assay--The plate incorporation assay is performed in the following way. To a sterile 13 x 100-mm test tube placed in a 43 C heating block are added:

1. 2.00 mL of 0.6% agar containing 0.6% NaCl, 0.05 mM biotin and 0.05 mM histidine
2. 0.10 mL of indicator organisms (about 10^8 bacteria)
3. 0.50 mL of metabolic activation mixture (if appropriate)
4. Appropriate amount of the test article (e.g., 20 mg/mL)

This mixture is stirred gently and then poured on plates containing about 25 mL of minimal glucose agar. After the top agar has set, the plates are incubated for 48 h at 37 C. The number of *his+* revertant colonies is counted using a BioTran II automated colony counter when possible. When accurate counts cannot be obtained (e.g., because of precipitate), the plates are counted manually using an electric probe colony counter.

10.2.2 Preincubation Assay--This assay is a minor modification of the standard plate incorporation assay (Yahagi et al. 1975). The bacteria, test substance and S-9 mix (or buffer) are added to a sterile 13 x 100-mm test tube. The tube is incubated with moderate agitation for 15 min at 37 C before the top agar, maintained at 43 C, is added. After the various substances are mixed, the mixture is poured onto 2% agar minimal glucose medium in a petri plate. Various incubation times, such as 5, 30 and 40 min can also be used.

10.2.3 Desiccator Assay--The standard Ames plate test is not entirely suitable for the testing of highly volatile chemicals. Low molecular weight volatile compounds, particularly those that are not hydrophilic, do not remain in the agar for sufficiently long periods at 37 C. It has been shown that the known mutagens/carcinogens methyl iodide and *bis*-(2-chloroethyl)-ether are not detected in the standard Ames protocol. However, their mutagenic activity can be detected easily when the plates are exposed to various concentrations of the chemical in a closed container such as a desiccator. For volatile compounds, the use of desiccators is recommended for experimental validity and for the protection of the workers.

Each standard 9-L desiccator holds no more than 12 plates, which can be arranged in either one of the following ways:

1. Duplicate plates for each of the five strains without or with one activation system (*10 plates per desiccator per dose level).

2. Duplicate plates for one strain without and with up to four activation systems (*10 plates per desiccator per dose level).

A total of five dose levels are used, in addition to the negative solvent control. The positive control chemicals are tested in the standard plate incorporation and preincubation assays.

For volatile liquids, the *Salmonella* plates are prepared as described for the assays in agar, but no test chemical is added. The plates, with the lids having been removed, are placed side by side on a perforated shelf in a 9-L desiccator. A known volume, such as 2, 5 and 10 mL of the test chemical, is added to a glass petri plate, which is then placed in the center of and attached to the bottom of the shelf. The desiccator is sealed and placed on a magnetic stir-plate in a room maintained at 37 C. A magnetic stirrer with vanes, placed in the base of each desiccator, ensures adequate dispersion of the chemical. After incubation for a known period, usually 8 h, the plates are removed from the desiccators, their lids are replaced and they are incubated at 37 C for an additional period up to 48 h. The number of *his*+ revertants is counted and recorded. The desiccator assay is illustrated on the next page. After each experiment, the desiccator is vented in a chemical fume hood overnight and then cleaned with soap and bactericide, followed by hexane and ethanol rinse.

11.0 Calculations

The calculations required for this protocol are the determinations of weight loss due to rotary evaporation, the percent of the initial sample weight partitioned into the acids, bases and neutrals fractions and the total recoverable weight resulting from fractionation.

Consider the following definitions:

A = Initial sample weight
B = Sample weight after rotary evaporation
C = Base fraction residue weight
D = Acid fraction residue weight
E = Neutral fraction residue weight

Then:

Weight loss due to Rotary Evaporation = $A-B$
% Weight loss due to Rotary Evaporation = $(A-B/A) \times 100$
% Weight of sample partitioned into base fraction = $(C/A) \times 100$
% Weight of sample partitioned into acid fraction = $(D/A) \times 100$
% Weight of sample partitioned into neutral fraction = $E/A \times 100$
% Total recoverable weight = $((C + D + E)/A) \times 100$

A data report sheet is illustrated in Figure 16.

Sample Identification No. ____________

Date: ____________

Analyst: ____________

Volume removed for Desiccator Assay: ____________

Weight Removed for Plate Incorporation and Preincubation Assays: ____________

% Weight Loss from Rotary Evaporation: ____________

Fractionation Sample Weight:

Base Residue Weight ____________

Acid Residue Weight ____________

Neutrals Residue Weight ____________

Total Recovery: $$\frac{\text{BASES + ACIDS + NEUTRALS}}{\text{SAMPLE WEIGHT}} \times 100 = \underline{\hspace{3cm}}$$

Figure 16. Data report sheet.

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SECTION 6

SOILS AND SEDIMENTS

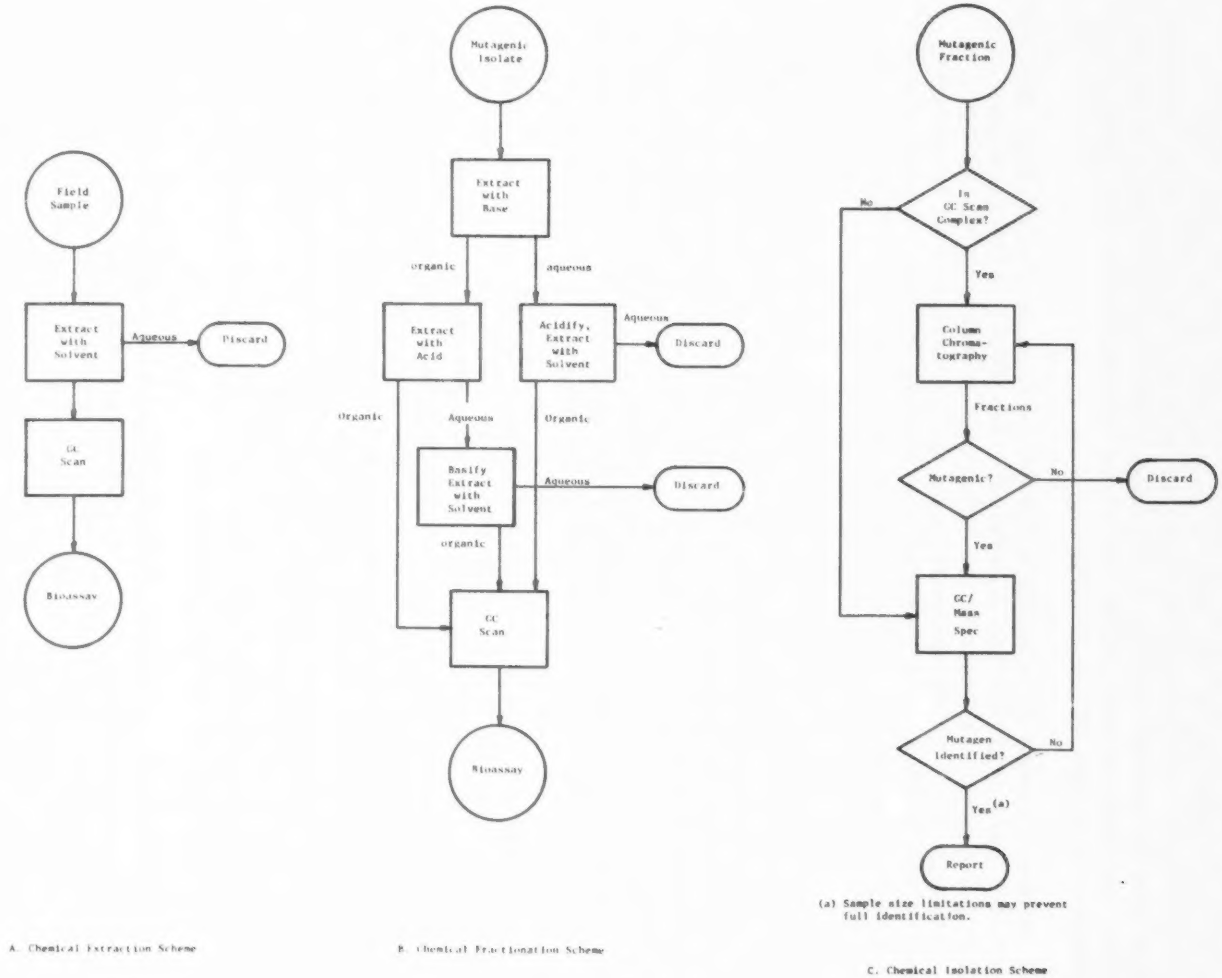
REVIEW OF THE LITERATURE

Introduction

Soil is the unconsolidated material on the earth's crust capable of supporting plant growth. Soil that has settled to the bottom of, or has been deposited by, a liquid is described as a sediment. Fertile soil is the sustenance of agriculture. Because environmental mutagens are frequently applied to soils either intentionally, accidentally or by atmospheric deposition, it is necessary to develop techniques that can be used to assess the mutagenic potential of both pristine and contaminated soils. Although a variety of biological test procedures have been developed and standardized (Ames et al. 1975, Scott et al. 1982, Liefer et al. 1981), no detailed protocols have been developed specifically for the preparation of soil and sediment samples for biological testing. The objectives of this paper are to provide a review of existing protocols and to develop a detailed protocol for the preparation of soil and sediment samples for biological testing.

There is very little information available describing studies where organic compounds from soil or sediment samples have been extracted for characterization in a biological test system. There have been a large number of earlier studies, however, involving the extraction of soil humic acid fractions. More recent research primarily describes the extraction of hydrocarbons (i.e, aliphatic and aromatic compounds) from contaminated soils or sediments. Only within the last four years has research been conducted using bioassays to characterize the mutagenic potential of soil (Goggelmann and Spitzauer 1982, Withrow 1982, Donnelly et al. 1983) and sediment (Kinae et al. 1981, Hirayama et al. 1981, Suzuki et al. 1982, Sato et al. 1983).

Bioassay-directed chemical analysis employs short-term mutagenicity assays to detect mutagenic fractions and to interpret the interactions of the components of a complex mixture; chemical analysis is used to characterize active constituents and to verify the absence of artifacts generated in the collection or extraction process. This biological and chemical characterization procedure includes three major phases: (a) chemical extraction to concentrate the compounds present and separate them from other associated compounds (Figure 17a); (b) chemical fractionation using techniques for separating acidic, basic and neutral organic compounds (Figure 17b); (c) final isolation of active fractions by chromatographic techniques and, if possible, identification of specific mutagenic compounds by mass spectral techniques (Figure 17c).


Field Sample
Extract with Solvent
Aqueous
Discard
GC Scan
Bioassay
Mutagenic Isolate
Extract with Base
organic
aqueous
Extract with Acid
Acidify, Extract with Solvent
Aqueous
Discard
Organic
Aqueous
Organic
Basify Extract with Solvent
Aqueous
Discard
organic
GC Scan
Bioassay
Mutagenic Fraction
Is GC Scan Complex?
No
Yes
Column Chroma-tography
Fractions
Mutagenic?
No
Discard
Yes
GC/ Mass Spec
Mutagen Identified?
No
Yes(a)
Report

(a) Sample size limitations may prevent full identification.

A. Chemical Extraction Scheme

B. Chemical Fractionation Scheme

C. Chemical Isolation Scheme

Figure 17. Chemical procedures, bioassay-directed chemical analysis.

Extraction

The initial extraction phase (Figure 17a) is designed to remove and concentrate the compounds in soil and sediment samples for subsequent mutagenicity testing. The compounds are generally extracted from a weighed amount of material (e.g., in a Soxhlet extractor) using ultrahigh purity organic solvents. The sample can then be redissolved in a solvent for biological testing or reextracted for further separation. The major compound classes that can be solvent extracted include aromatic halocarbons, aromatic hydrocarbons, chlorinated pesticides, nitrogen heterocyclics, halogenated ethers, phenols, anilines and quinolines.

These various classes of compounds may be solvent extracted from soil and sediment samples using the various laboratory techniques which have been previously applied to the extraction of industrial wastes or environmental samples. Although the Soxhlet extraction is the procedure most frequently used, Donnelly and Brown (1981) compared the Soxhlet extraction with a more rapid extraction using a blender and found no appreciable difference in the mutagenic potential of the extracted material using either method.

The Soxhlet extraction is designed for use with finely-divided solids that are readily dispersible in the extracting solvent (Warner et al. 1983). The Soxhlet technique is limited by the increased chemical reactivity which can occur at the elevated temperature of the refluxing solvent and the increased extraction time which is required if the procedure is conducted overnight (Warner et al. 1983). An advantage of the blender extraction for soil and sediment samples is that it works very well for dispersing particulate in the extracting solvent to promote extraction efficiency (Warner et al. 1983). A third procedure uses a sonic probe to disperse the sample in an extracting solvent. The sonification procedure is not as effective as the blender for reducing the size of solid particles, although it is particularly advantageous for extracting sandy sediments which may cause excessive wear of blenders (Warner et al. 1983). Adams (1983) compared the efficiency of the sonic, blender and Soxhlet procedures for extracting aza-arenes. She observed that the Soxhlet and blender procedures provided acceptable reproducibilities, while the sonic procedure gave poor reproducibilities for many compounds (Adams 1983). The procedure which is ultimately selected for the initial extraction of soil and sediment samples will be selected according to the characteristics of the materials present in the sample. The blender extraction should be adequate for the majority of samples; however, it may be advantageous to use the Soxhlet extraction if a sample contains materials which could be resistant to solvent extraction.

The solvent selected to extract organic compounds from soil is usually dictated by the characteristics of the compounds present. However, additional restrictions will apply to the selection of solvents prior to biological testing. Two main factors will influence the selection of a soil extraction solvent system prior to biological testing. First, the solvent must remove both toxic and growth-stimulating substrates that could interfere with the bioassays. These will include toxic compounds which could inhibit the detection of mutagenic compounds and growth substrates, such as histidine, that could mask the detection of weak mutagens. Brown et al. (1984b) reduced interference due to toxic substances by separating a complex mixture into three fractions, thus isolating the toxic compounds. Interference due to histidine can be reduced by using a bioassay without histidine interaction, such as A. nidulans (Scott et al. 1982), or by eliminating histidine from the test sample through a solvent or sorbent extraction procedure such as those suggested by Aeschbacher et al. (1983). The solvent selected to extract samples for mutagenicity assays should also avoid generating artifacts which increase or decrease the mutagenic potential of the soil extract. Solvents or their contaminants may alter the mutagenic potential of a test substance during the extraction procedure through oxidation or through the formation of radicals (Aeschbacher et al. 1983). Thus, the extraction and fractionation procedure used to concentrate samples for mutagenicity testing should employ solvents and procedures that cannot chemically modify the test material.

A variety of solvents have been employed to extract organic compounds from soil for chemical analysis. For the extraction of humic substances from soil, Ogner and Schnitzer (1970) used benzene and ethyl acetate, while Cifrulak (1969) used a benzene-methanol-acetone (2:1:1) mixture. Several studies have used solvent extraction to determine the oil content of soil. Jobson et al. (1974) used n-pentane, while Jensen (1975) used carbon tetrachloride, and McGill and Rowell (1980) used methylene chloride. Methylene chloride was also used by Wakeham (1979) to extract aza-arenes from sediment. Carey and Gowen (1978) extracted polychlorinated biphenyls from soil with a 3:1 mixture of hexane:isopropanol. Each of these studies was concerned with the efficient extraction of organic compounds from soil, and none addressed the limitations associated with extractions for biological testing.

Only recently has research been conducted where soil or sediment samples were extracted for analysis in a biological test system. There have been two publications reporting on the mutagenic activity of agricultural soil. Goggelmann and Spitzauer (1982) used a 2:1 hexane:acetone system to extract soil. Dichloromethane was used by Brown et al. (1984a) to extract three agricultural soils for mutagenicity testing. A study examining the mutagenicity of sediment samples by Kinae et al. (1981) obtained two primary fractions by successive extractions with diethylether and methanol. Kinae et al. (1981) observed a higher level of mutagenicity in one of the ether extracts, although neither solvent gave consistently higher yields of hydrocarbons. Sato et al. (1983) also used ether to extract sediment samples. However, prior to biological testing, Sato et al. (1983) further fractionated the ether extract using a series of solvents. Any one or a combination of an assortment of solvents may be used to extract organic compounds from soil or sediment samples. If the solvent will be evaporated prior to biological testing, any solvent can be used, provided it does not generate artifacts and does remove interfering substrates.

The USEPA (1982) recommends dichloromethane, and the results of Brown et al. (1984a) indicate that dichloromethane does not cause interference. Although a 1:10 dichloromethane:dimethyl sulfoxide solvent mixture does not influence the mutagenicity of diagnostic mutagens in the Salmonella assay, this solvent mixture can affect the results of other bioassays (Brown et al. 1984b). Thus, a more limited group of solvents can be used to dissolve the dried residue prior to biological testing. Organic solvents which are compatible with the *Salmonella* test include dimethyl sulfoxide, glycerol formal, dimethyl formamide, formamide, acetonitrile, 95% ethanol, acetone, dimethyl glycol, dimethyl ether, 1-methyl-2-pyrrolidinone, p-dioxane, tetrahydrofurfuryl alcohol and tetrahydrofuran (Maron et al. 1981). Abbondandolo et al. (1980) observed that ethylene glycol, 2-methoxyethanol and methanol were also compatible with mutagenesis test systems. These results indicate that dichloromethane should be the primary solvent system used for extraction, while dimethyl sulfoxide or other solvents compatible with bioassays can be used as a secondary solvent to redissolve the residue after the extracting solvent has been evaporated. Therefore, the optimum procedure for routine extractions would be a blender extraction using dichloromethane, while for certain compounds which are resistant to this procedure, alternate procedures or solvents should be investigated.

Fractionation

The majority of studies reporting on the mutagenic potential of a complex mixture have employed at least a primary fractionation scheme to separate active fractions. Organic constituents that are amenable to evaporative-concentration techniques may be separated in classes by methods based on their functional group acid-base reactions (Adams et al. 1983). The liquid-liquid acid-base extraction method is based on the acidity constants (pK_as) of organic functional groups within the compound's molecular structure. Compounds which reside in the organic phase at low pH, low pK_as, are characterized as acidic. Compounds which reside in the organic phase at high pH are characterized as basic, and compounds which exhibit no change in solubility at an altered pH are neutral. Liquid-liquid extraction can be used to separate a complex organic mixture into acid, base and neutral fractions by selectively adjusting the pH of the aqueous phase. Brown et al. (1984b) employed this procedure to separate the dichloromethane extract of hazardous waste and waste-amended soil into three primary fractions (acid, base and neutral). Sato et al. (1983) also used a liquid-liquid acid-base extraction to fractionate the ether extract of sediment samples. A similar procedure is described by Epler et al. (1978) and Guerin et al. (1980) for the separation and identification of mutagenic constituents of petroleum substitutes. Guerin et al. (1980) used diethylether to obtain a primary extract with an acid-base extraction to further separate the primary extract. Pelroy and Peterson (1981) obtained seven fractions of shale oil for mutagenicity testing using isooctane coupled with a liquid-liquid extraction. Following solvent extraction of the acid and base fractions, Pelroy and Peterson (1981) extracted the isooctane phase with dimethylsulfoxide to obtain neutral and polycyclic aromatic hydrocarbon subfractions. Thus, solvent extraction can be used to extract organic material from soil and sediment samples, and this primary extract can be further separated into three to six subfractions by a liquid-liquid acid-base extraction.

Isolation

Additional separation of the three primary fractions generated through acid-base extraction may be required in some cases in order to isolate the mutagenic components of a complex organic mixture. Chemical isolation procedures generally employ chromatographic techniques coupled with a separation based on the polarity of the extracting solvents. The most simple technique uses a silica gel column and is described by Warner (1976). Brown et al. (1984b) employed this procedure to bioassay four neutral subfractions of hazardous waste. Brown et al. (1984b) showed that subsequent elutions with petroleum ether, followed by two elutions with dichloromethane:petroleum ether (1:4) and a final elution with dichloromethane, yielded a saturate, aromatic and two condensed ring fractions. Epler et al. (1978) used Florisil (registered trademark, Floridin Company) to separate the neutral fraction of crude oil for biological testing. The procedures of Bell et al. (1969) were used to elute fractions from the Florisil column with hexane, hexane:benzene (8:1), benzene:ether (4:1) and methanol. Sephadex LH-20 (Pharmacia Fine Chemicals) gel has also been used to isolate bioactive components of a complex mixture. Jones et al. (1977) describe a three-step fractionation procedure. The mixture is first separated into lipophilic and hydrophilic fractions using Sephadex LH-20 gel swollen with methanol/water and eluted with hexane. The lipophilic fraction can be further separated into polymeric, sieved and hydrogen bonding constituents using a column of LH-20 swollen and eluted with tetrahydrofuran. In the final step, the gel is swollen and eluted with isopropanol to separate the sieved fraction into aliphatic, aromatic and polyaromatic fractions. Toste et al. (1982) followed partition chromatography using Sephadex LH-20 with high-performance liquid chromatography (HPLC) to facilitate the organic and biological analyses of synfuels. Donahue et al. (1978) also used HPLC to isolate mutagenic impurities in carcinogens and noncarcinogens. A technique developed by Bjorseth et al. (1982) employed thin-layer chromatography plates to separate the components of complex mixtures and to evaluate their mutagenic potential directly by means of the Salmonella assay. Chromatographic techniques are useful for the separation of the components of a complex mixture when chemical identification is required or when the mutagenic constituents are present in dilute concentrations. There are two primary disadvantages associated with generating a large number of fractions using chromatographic techniques. First, the expense of conducting multiple bioassays will be substantially increased when compared to the cost for testing three to six primary fractions. In addition, extensive separation of a complex mixture will make it difficulte to interpret the interactions that will influence the toxicity of the mixture as a whole. However, chromatographic fractionation may be necessary when toxic substrates interfere with the bioassay or when mutagenic substrates are present in dilute quantities.

All soil and sediment samples will require at least a preliminary extraction to obtain a sample for mutagenic characterization. However, extreme caution should be exercised when interpreting the test results of a fractionated mixture. In order to obtain the most accurate characterization, each successive extract or fraction should be tested in at least one biological test system. If the original extract is mutagenic, it should be separated into acid, base and neutral fractions. Those fractions giving a positive response in the bioassay will be further isolated using liquid/liquid separation and/or chromatographic techniques, followed by a retest in the bioassay.

At each stage of isolation, only those fractions showing mutagenic activity will undergo further chemical separation. Using this type of extraction and analytical protocol will make it easier to evaluate the interactions of the components of a complex mixture.

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SOILS AND SEDIMENTS WORKGROUP REPORT

Workgroup Tasks

Summary of Tasks--

The following questions for performing a soil or sediment sample for Ames/Salmonella testing were considered:

1. Effect of soil pH on efficiency of extraction of organics.
2. Choice of solvent for extracting soil.
3. Need to demonstrate efficiency of extraction (using spiked samples).
4. Storage of sample (temperature and time).
5. Storage of extract.
6. Method of extraction.
7. Restriction of assay.
8. Subsequent sample extraction following initial Ames test on crude extract.
9. Need for replicate samples and assay.
10. Sample handling to prepare.
11. Proper negative control.

Key Peer Review Comments--

There are no unresolved peer review comments.

Summary of Workgroup Progress--

Major questions considered and rationale for choice:

1. While it may be desirable to choose different extraction solvents based on the possible soil contaminants, this information will not be available for most samples. Therefore, CH_2Cl_2 was chosen as the most "universal" solvent.

2. The extraction procedure should use the Soxhlet method routinely, since this procedure is well-known and widely used. However, other methods were given as alternatives and may be more efficient. We felt that the burden was on the principal investigator to demonstrate with spiked soil samples that the alternative methods were as efficient as the Soxhlet.

3. Fractionation of extract following initial Ames test was dependent on observation of a toxic nonmutagenic result. Two methods are available: acid/base/neutral techniques, as previously published, and HPLC method, as described by Dr. Tabor. We felt the principal investigator should have the freedom to choose the fractionation scheme.

Summary of Key Workgroup Discussions

Major Consensus Opinions--

There was uniform agreement that labs should use spiked samples to demonstrate their ability to extract organics from soil. We listed several methods that could be used to determine efficiency of extraction.

We also did not wish to restrict the principal investigator to one extraction method; thus, we listed the Soxhlet method as the primary one, but gave an alternative method that ultimately may be faster. Similarly, the procedure to further fractionate the extract following the initial Ames test was left open. We listed the most widely used method, acid/base/neutral techniques, and then a second method, HPLC. This HPLC method may ultimately be the most useful for the future, and we felt it was important to have it listed as an alternative procedure to be used at the discretion of the researcher. The question of internal standards or a universal soil blank for negative controls was asked in the general meeting. We concluded that the use of an internal standard for a sample to be tested in a bioassay is inappropriate, since it would obscure the analysis of the sample itself. A universal soil blank is not needed as a negative control; proper characterization of the solvent negative control is sufficient as a "blank."

Other Data or Information Requirements

An important information gap that we found in developing this protocol was quantitative data on the stability of organic pollutants in soil under various storage conditions. To bridge this gap, a research program needs to be initiated to measure the degradation of a variety of compounds spiked into soil under various storage conditions. The effects of storage temperature (-4 C to -80 C), soil moisture, soil type and storage time (1, 3, 6, 9 and 12 months) should be determined as a minimum. Another problem that could be addressed in this study is the stability of the compounds in autoclaved soil. That work is important because totally stable soil performance evaluation samples are not presently available.

Another information gap is in the area of solvent extraction of mutagens from soil. The recoveries of a variety of organic compounds of different polarities and/or functional groups should be extracted from several soil types adjusted to pH 4, 5, 7 and 8 with methylene chloride only, methylene chloride followed by methanol, a combination of methylene chloride and methanol (9:1 v/v) or other solvent mixtures or sequences suitable for extraction of soils/sediment. The recoveries of the compounds and the suitability of the different solvent systems for compounds of different polarity should be determined. In addition, the effect of soil pH on the extraction of various compounds should be quantitated and optimized.

A final area of research that needs to be investigated is the fractionation of crude extracts. The effect of acid and base fractionation on a variety of mutagenic extracts could be compared side by side with the HPLC fractionation technique discussed by Dr. Wilson Tabor at this meeting. The sum of the (revertants-background)/gram soil for each of the techniques could be compared, and the claim that acid-base fractionation destroys mutagenic activity could be documented. If the claims about HPLC fractionation are supported by these studies, additional work should be done investigating reversed phase flash chromatography as an inexpensive alternative to routine HPLC fractionation.

PROTOCOL FOR THE PREPARATION OF SOIL AND SEDIMENT SAMPLES FOR MUTAGENICITY TESTING

1.0 Scope and Application

1.1 Application--

The following protocol has been developed for the preparation of soil and sediment samples for mutagenicity testing. This protocol is designed to provide samples which accurately represent the mutagenic potential of the soil or sediment sample initially extracted and, if necessary, sufficiently fractionate the original material to isolate bioactive materials. Fractionation should only be required if the toxicity of the crude extract prevents determination of the mutagenic potential. Included in this protocol are procedures for sample preparation, extraction and fractionation. The use of standardized procedures for mutagenicity testing will increase the accuracy of the bioassay results and allow interlaboratory comparisons to be made.

1.2 Method Detection Limits--

The method detection limit will be specific for each compound and each biological test procedure. In addition, the interactions of the components of a complex mixture will also affect the detection limits of a bioassay. Thus, sensitivity should be determined for each experiment using solvent and positive controls.

1.3 Limitations--

This protocol includes procedures for preparing solvent-extractable organic compounds for mutagenicity testing. The protocol does not include procedures for the preparation of inorganic (i.e., heavy metals) or volatile organic constituents, which will be removed or isolated in the preparation of the solvent-extractable organic constituents.

2.0 Summary of Method

This protocol describes primary and optional procedures for the isolation and fractionation of soil and sediment samples for mutagenicity testing. The protocol includes a Soxhlet, blender and sonification procedure for solvent extraction of organic compounds. In addition, two procedures, a liquid-liquid, acid-base extraction and an HPLC extraction, are provided for the fractionation of the solvent-extractable organic compounds.

3.0 Definitions

a. Soil - The unconsolidated material on the earth's surface that is less than 2 mm in diameter and capable of supporting plant growth.

b. Sediment - Soil material deposited and remaining in an aquatic environment.

c. Fractionation - The separation of solvent-soluble compounds from a bulk matrix.

d. Extraction - Manipulation of chemical and/or physical conditions within a sample matrix which effectively isolates and enriches a specific class of compounds sensitive to the manipulation.

4.0 Interferences

Two types of interferences can be anticipated in a mutagenicity assay. Interference due to growth substrates (e.g., histidine) should be removed using the extraction procedure (Sections 7-9), while interference caused by toxic compounds may be reduced by fractionation procedures (Sections 10 and 11).

5.0 Safety

This procedure is to be used for the collection and preparation of soil or sediment samples for mutagenicity testing. All samples that are collected should be treated as biohazardous material. Thus, appropriate safety precautions must be taken.

a. Samples from an area where a spill of a known or suspected high-level mutagen has occurred should be taken by properly trained personnel wearing disposable full-coverage suits and masks. Bareis et al. (1983) suggest that in areas where the anticipated contaminant levels are more than 5 ppm above background, individuals should also wear a self-contained breathing apparatus. These precautions will not be necessary in gathering routine samples from areas when no release of mutagens has occurred.

b. The threads of the sample collection bottles should be wiped free of any residue before sealing the jar. After the jar is sealed, it should be wiped clean and enclosed in a plastic bag before transport to the lab or storage facility. Materials used to wipe the jars should be disposed properly.

c. Sample bottles should be removed from the plastic bags in a high draft hood and examined to determine that they are not cracked or broken.

d. All manipulations involved in the extraction or fractionation of samples should be performed in a high draft hood.

e. The solvent exchange of extracts and fractions into DMSO and the manipulation of the DMSO solutions must be handled in a high draft hood. In addition, the DMSO solutions must be handled with two pairs of gloves. Special precautions are necessary with these potentially mutagenic samples because of the unique biological transport of DMSO.

f. Spiking of soil samples with mutagenic compounds that serve as positive controls is to be done in a hood with great care. Two pairs of gloves should be worn, and the dissolved mutagens should be applied to the soil at close range in order to minimize drift.

g. All samples, extracts or fractions must be handled with caution and, until proven otherwise, they should be treated as mutagens.

h. All contaminated material should be handled and disposed following proper procedures for the disposal of hazardous waste.

6.0 Sample Collection, Preservation and Handling

Collection, preservation and handling of soil and sediment samples for biological testing must be performed in such a manner that mutagenic activity is neither lost nor generated. It is especially important that samples of soil or sediment collected for mutagenicity testing provide an accurate representation of the sampling location. Sampling procedures are discussed in Peterson and Calvin (1965) and deVera et al. (1980). The USEPA (1980) recommends a one-quart, wide-mouth, screw-cap, glass bottle with a Teflon lid liner for storing soil and sediment samples. At the time of collection, the bottle should be filled nearly to the top with the soil or sediment sample. If the sample is collected from below water, the threads and sealing surfaces should be washed off with the sample water. Sediment samples should be "topped off" with sample water and sealed with the Teflon-lined screw cap. Soil and sediment samples should not be air or oven dried before storing at 4 C. Samples stored more than 48 h should be stored at -10 C. Care should be taken with anaerobic samples to avoid contact with oxygen. A sufficient sample should be collected to provide adequate residue for the needs of the bioassay and a reserve sample. In most cases, approximately 500 g should be adequate for a soil contaminated by oil or other organic materials. For uncontaminated soils or soils with a high moisture content, 2 to 3 kg of sample may be required.

Soil and sediment samples to be analyzed for mutagenic potential should be labeled by procedures prescribed for handling hazardous waste. Primary particles greater than 2 mm in diameter must be removed by sieving as a first step in sample preparation. It may be necessary to break aggregates by dicing or crushing to facilitate subsampling. Samples must be homogenized and properly subsampled to assure that the results are not biased by spot concentrations in the soil. This can best be done by repeatedly quartering the samples to avoid the biased selection of a given particle size. Quartering is done by spreading the sample on a clean piece of paper (typically 60 cm by 60 cm). The centers of opposite edges of the material are then raised, separating the sample in half. The centers of the other two opposite edges are then raised, dividing the sample into quarters. Three quarters of the sample is returned to the sample container, and the remaining quarter is requartered until the desired sample size is achieved. A duplicate sample must be used to determine the moisture content in order to allow reduction of the data to revertants per dry weight of the solids.

Sediment samples may frequently contain large amounts of water which need to be separated from the soil prior to extraction. The water can be separated from the soil by filtration of the sample under a nitrogen blanket or by centrifuge. The weight of the soil sample should be determined before and after the water is removed. If the water sample is to be analyzed, it should be prepared according to the procedures described for wastewater.

7.0 Extraction Protocol (Soxhlet)

Three procedures for the extraction of soils and sediments will be described. While the Soxhlet method (USEPA 1982) is the preferred method and should be used when a limited number of samples are to be tested, the blender method (Donnelly and Brown 1981, Brown et al. 1982, Adams 1983, Brown et al. 1984) and the sonification method (Warner et al. 1983) are less complicated and time-consuming. These alternate procedures may be used if data comparing the extraction efficiency with the Soxhlet method are presented.

7.1 Apparatus and Equipment--

a. Soxhlet extractor - 40-mm ID, with 500-mL round-bottom flask.

b. Kuderna-Danish (K-D) apparatus with a three-ball Snyder column or vacuum rotary evaporator with chilled solvent receiving flask.

c. Chromatography column - Borosilicate glass, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium. This is used as a drying column.

7.2 Reagents and Consumable Materials--

a. An appropriate solvent or solvent mixture of HPLC grade. In most instances, dichloromethane can be used; subsequent extraction with methanol may be necessary to recover polar compounds.

b. Anhydrous sodium sulfate, ACS (purified by heating at 400 C for 4 h in a shallow tray).

7.3 Procedure--

a. Mix 10 g of soil or sediment sample with 10 g of anhydrous sodium sulfate and place in a glass extraction thimble. Mix thoroughly with a glass rod. If any problems are encountered, e.g., the sample clogs the thimble, an alternative is to place a plug of glass wool in the extraction chamber, transfer the sample into the chamber and then cover with another plug of glass wool.

b. Place thimble and glass rod in the Soxhlet extractor. Assemble condensers and boiling flasks containing 300 mL of solvent and a boiling stone (solvent-extracted). Apply heat to the boiling flask and adjust the temperature to give six flushings per hour (caution: avoid excess temperature). Extract the sample for 16 h.

c. After the extraction is complete, the solvent extract should be cooled and then filtered and dried by passing it through a 10-cm column of solvent-washed sodium sulfate.

d. The procedure used for solvent reduction will be the same for all extraction procedures and is described at the end of this protocol.

8.0 Extraction Protocol (Blender)

8.1 Apparatus and Equipment--

a. Explosion-proof blender, accommodating stainless steel or borosilicate glass container with 1.2-L capacity.

b. Kuderna-Danish apparatus with a three-ball Snyder column or vacuum rotary evaporator with a chilled solvent receiving flask.

c. Chromatography column - Borosilicate glass, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium. This is used as a drying column.

8.2 Reagents and Consumable Materials--

a. An appropriate solvent or solvent mixture of HPLC grade. In most instances, dichloromethane can be used; subsequent extraction with methanol may be necessary to recover polar compounds.

b. Anhydrous sodium sulfate, ACS (purified by heating at 400 C for 4 h in a shallow tray).

8.3 Procedure--

a. Mix 25 g of soil or sediment sample with 25 g of anhydrous sodium sulfate and place in blender jar.

b. Add approximately six volumes (150 mL) of the extracting solvent to the blender jar and screw cover down tightly.

c. Blend mixture for 30 sec in a fume hood. Decant solvent and repeat extraction twice with 75 mL of fresh solvent or until extracting solvent remains colorless.

d. Combine solvent extracts and then filter and dry by passing through a 10-cm column of solvent-washed sodium sulfate.

e. The procedure used for solvent reduction will be the same for all extraction procedures and is described at the end of this protocol.

9.0 Extraction Protocol (Sonification)

9.1 Apparatus and Equipment--

a. Sonic agitator equivalent to the Heat Systems Ultrasonic 375 W with a 12-cm x 1.25-cm probe.

b. Kuderna-Danish apparatus with a three-ball Snyder column or vacuum rotary evaporator with chilled solvent receiving flask.

c. Chromatography column - Borosilicate glass, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium. This is used as a drying column.

9.2 Reagents and Consumable Materials--

a. An appropriate solvent or solvent mixture of HPLC grade. In most instances, dichloromethane can be used; subsequent extraction with methanol may be necessary to recover polar compounds.

b. Anhydrous sodium sulfate, ACS (purified by heating at 400 C for 4 h in a shallow tray).

9.3 Procedure--

a. Mix 25 g of soil or sediment sample with 50 g of anhydrous sodium sulfate and place in a 250-mL beaker.

b. Add approximately six volumes (150 mL) of the extracting solvent and agitate with a 375 W Sonicator with a 12-cm by 1.25-cm probe positioned 1 cm below the surface of the solvent for 3 min on a 50% duty cycle with a power input of 50% full scale.

c. Decant the solvent and remix the soil or sediment sample.

d. Repeat the extraction twice with 150-mL volumes of solvent or until the extracting solvent remains colorless.

e. Combine solvent extracts and then filter and dry by passing through a 10-cm column of solvent-washed sodium sulfate.

f. The procedure used for solvent reduction will be the same for all extraction procedures and is described at the end of this protocol.

10.0 Solvent Reduction Procedure

a. Although a variety of concentration techniques may be used, Adams et al. (1982) obtained the best recovery using a rotary evaporator with the solvent-receiving flask immersed in an ice bath. Thus, procedures for both a K-D and rotary evaporator concentration will be provided. The solvent extract should be collected in a 500-mL K-D flask fitted with a 90-mL graduated concentrator tube. The extractor flask and sodium sulfate column should be washed with 100 to 125 mL of the extracting solvent.

b. Concentration using a K-D apparatus is accomplished by adding one to two solvent-treated boiling chips to the flask and attaching a three-ball Snyder column. Prewet the Snyder column by adding about 1 mL of solvent to the top. Place the K-D apparatus on a steam or hot water bath so that the concentrator tube and the entire lower rounded surface of the flask is bathed in hot water or vapor. Adjust the vertical position of the equipment and the water temperature to complete the extraction in 15 to 20 min (approximately 45 C). At the proper rate of distillation, the balls of the column will actively chatter, but the chamber will not flood. When the volume of the liquid is less than 10 mL, remove the K-D apparatus and allow to drain for at least 10 min while cooling.

c. If a rotary evaporator is to be used, transfer the solvent extract to a 500-mL rotary evaporator flask. The extractor flask and sodium sulfate column should be washed with 100 to 125 mL of the extracting solvent. Adjust evaporator flask water bath temperature to 45 C, circulate chilled coolant through condenser, attach vacuum port to aspirator or other vacuum source and place solvent-receiving flask in an ice bath. Reduce solvent volume to less than 20 mL on rotary evaporator and transfer with rinsing to a sample collection tube.

d. An aliquot of the solvent extract may be removed for gravimetric analysis of solvent-extractable organic carbon or for fractionation prior to the final concentration step. The final concentration step will employ a solvent exchange using DMSO. The volume of the initial solvent extract should first be carefully reduced to 1 mL at 40 C under a gentle stream of nitrogen. Because the solvent evaporates rapidly, it is important that this operation be done under constant surveillance to ensure that the volume is not reduced below 1 mL. It is also necessary to warm the samples slightly, either by hand or water bath at $\leq$40 C, to prevent condensation of atmospheric moisture in the sample by evaporative cooling.

e. Add 1 mL of DMSO to the sample and mix by gentle agitation. The total sample volume is reduced under a stream of nitrogen to 1.5 mL. Another 1 mL of DMSO is added and mixed, and the volume is reduced to 2.25 mL. The exchange is repeated with another 1 mL of DMSO, and the volume is reduced to a final volume of 3 mL. Other volumes of DMSO may be used if 3 mL of DMSO does not give a suitable sample concentration.

11.0 Fractionation Protocol

The least complex and most widely accepted protocol for fractionation of an organic extract employs a liquid-liquid extraction to partition acid, base and neutral constituents (Figure 18). This procedure is recommended for evaluating solid waste by the USEPA (1982) and Adams et al. (1983) and was used by Brown et al. (1984b) to isolate mutagenic fractions from waste-amended

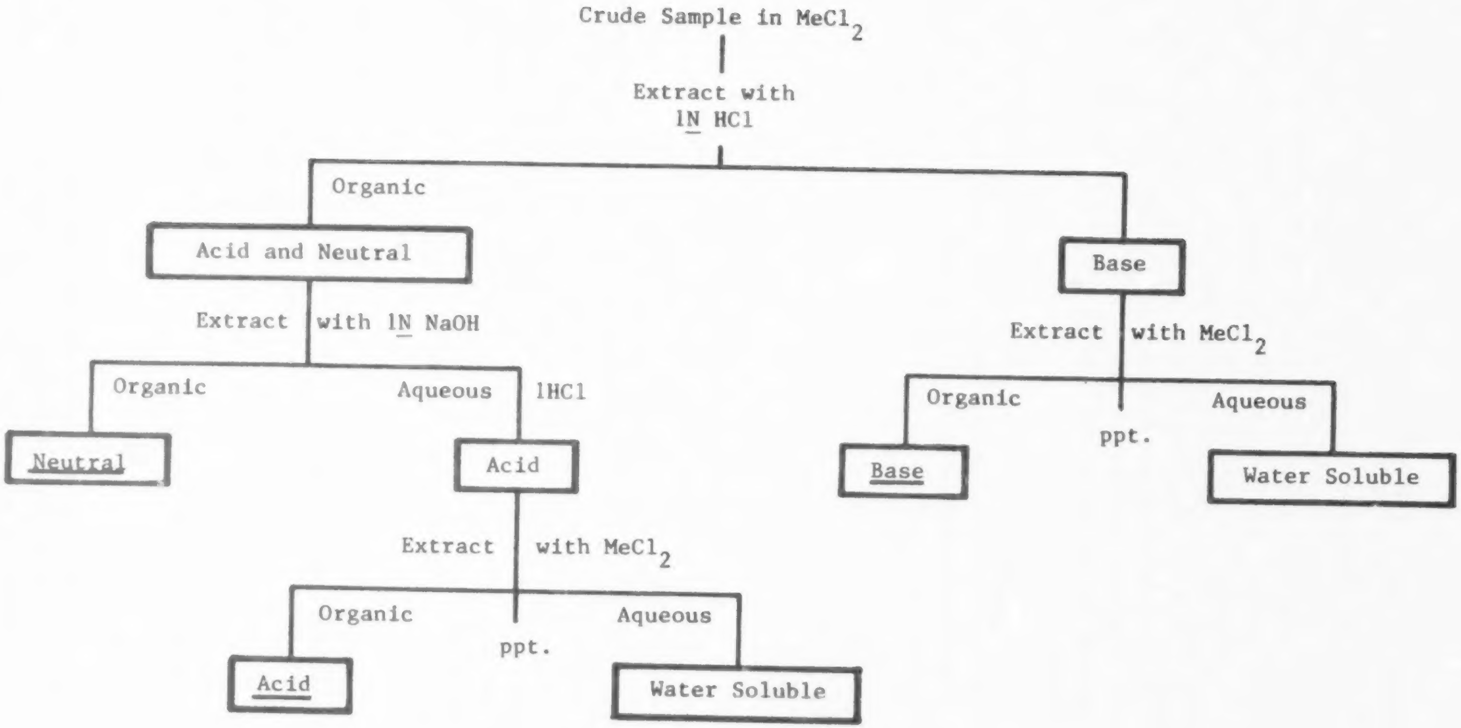
Crude Sample in $MeCl_2$
Extract with
1N HCl
Organic
Acid and Neutral
Extract with 1N NaOH
Organic
Neutral
Aqueous
1HCl
Acid
Extract with $MeCl_2$
Organic
Acid
ppt.
Aqueous
Water Soluble
Base
Extract with $MeCl_2$
Organic
Base
ppt.
Aqueous
Water Soluble

Figure 18. Fractionation scheme for soil and sediment samples.

soil. An optional procedure which employs HPLC to fractionate samples is also included for samples where it is desired to avoid the risk of hydrolyzing mutagenic compounds with NaOH.

11.1 Apparatus and Equipment--

a. Separatory funnel with a Teflon stopcock (2,000 mL).

b. Kuderna-Danish apparatus with a three-ball Snyder column or vacuum rotary evaporator with a chilled solvent receiving flask.

c. Chromatography column - Borosilicate glass, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium.

d. Evaporator flasks as appropriate for collecting fractions.

11.2 Reagents and Consumable Materials--

a. Sodium hydroxide - (ACS) 1N in distilled water (chilled to 4 C).

b. Hydrochloric acid - (ACS) 1N prepared by mixing concentrated HCl and distilled water (chilled to 4 C).

c. An appropriate solvent or solvent mixture of HPLC grade. In most instances, dichloromethane can be used.

d. Anhydrous sodium sulfate - (ACS) purified by heating at 400 C for 4 h in a shallow tray.

e. Distilled water - chilled to 4 C.

f. pH test paper providing readings over entire pH range from 0-14.

11.3 Procedure--

a. Dissolve 10 g or the extract in 30 mL of solvent. In most instances, the solvent will be dichloromethane.

b. Place sample in a separatory funnel.

c. Adjust the pH to 1-2 with sulfuric acid (approximately 100 mL). Partition the sample into the two phases by shaking the funnel for 1 min with periodic venting to release pressure (point funnel away from the analyst).

d. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solid layer, the analyst must employ a mechanical device to complete the phase separation. The optimum mechanical technique depends on the sample, but may include stirring, filtration of the emulsion through glass wool or centrifugation.

e. The organic layer will be the lower phase. This phase will contain the acid and neutral fractions. Collect the upper phase in a flask and label. The upper phase contains the base fraction.

f. Reextract the solvent layer twice more with 1N HCl at pH 1-2 (approximately 50 mL each). Combine the aqueous base extracts and label.

g. Adjust the pH of the aqueous layer obtained in Steps e and f to pH 12-13 by the addition of 1N NaOH to the base fraction and 200 mL of dichloromethane.

h. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete the phase separation.

i. The organic layer will be the lower phase. This phase will contain the base fraction in dichloromethane. Collect the lower phase and label. Add 100 mL of dichloromethane and repeat extraction to collect a second base fraction in dichloromethane, which is added to the first.

j. Adjust the pH of the solvent layer obtained in Steps e and f to pH 12-13 by the addition of 1N NaOH (approximately 200 mL). Partition the sample into two phases by shaking the funnel for 1 min with periodic venting to release pressure (point funnel away from the analyst).

k. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete the phase separation.

l. The organic layer will be the lower phase. This phase will contain the neutral fraction. Collect the upper phase in a flask and label. The upper phase contains the acid fraction.

m. Reextract the solvent layer twice more with 1N NaOH (approximately 50 mL each). Combine the aqueous acid extracts.

n. The organic layer will contain the neutral fraction.

o. Adjust the pH of the aqueous layer obtained in Steps l and m to pH 1-2 by the addition of 1N HCl and extract the acid fraction with 200 mL of dichloromethane.

p. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete phase separation.

q. The organic layer will be the lower phase. This phase will contain the acid fraction in dichloromethane. Collect the lower phase in a flask and label. Add 100 mL of dichloromethane and repeat the separation to collect a second acid fraction in dichloromethane, which is added to the first.

r. The dichloromethane fractions obtained in Step i (base fraction), Step n (neutral fraction) and Step q (acid fraction) are concentrated separately using the procedures described in Section 10. The corresponding aqueous fractions and any precipitate remaining represent additional fractions which, in most instances, will not be tested in the bioassay.

11.4 Fractionation Procedure (HPLC)--

An alternate procedure which can be used to fractionate the solvent extract of soil and sediment samples employs an isocratic gradient on an HPLC. This procedure is less likely to hydrolyze organic compound than the acid-base extraction and is described in detail in the section on Drinking Water.

12.0 Calibration

Due to the variety of matrix conditions exhibited by soil and sediment samples, verification of the solvent extraction efficiency must be conducted on each batch of soil or sediment samples. The procedure outlined below is designed to meet the above objective:

a. Select 25 g of sample typical of the lot to be assayed, as described above. Cool the sample and store in a dry place.

b. Prepare a solution of 250 mg/mL 2-nitrofluorene and 2-aminoanthracene in methylene chloride.

c. Spike the matrix with 1 mL of spike solution and allow to stand for 24 h. Extract the matrix according to the procedure in Section 7. Quantify the extract for the spike compounds. Alternatively, extraction recoveries can be measured by another suitable technique that has been validated. Two possibilities are (1) determination of the amount of deuterated priority pollutant surrogates by GC/MS or (2) determination of ^{14}C-labeled mutagens by scintillation methods. A recovery of 80% of each spike compound is considered acceptable. Below 80% recovery indicates a potential problem. For some difficult cases, 70% may be acceptable; however, 70% is not acceptable on a routine basis.

Listed below are several suggestions for examination when the extraction efficiency is below specification:

a. Recalibrate the HPLC and rerun the analysis.
b. Reexamine dilutions, weighing, etc.
c. Start from the beginning with the same matrix.

d. Start from the beginning, but choose a different sample from the same series.

e. Start from the beginning with a matrix from a different source.

Once the presence and quantity of the spikes have been established, the extract can be used to verify the detection limit of the chosen mututation assay. Note: Storage of this extract is subject to the same restrictions as the sample extracts. Standard samples of mutagens should be stored for no longer than these solutions have been demonstrated to be stable.

13.0 Quality Control

All laboratories preparing or testing soil and sediment samples should follow a Quality Assurance/Quality Control program. The Quality Assurance program ensures that all laboratory or field procedures are performed in a scientifically sound manner. The Quality Assurance program should be based on the guidelines set forth in "Good Laboratory Practice Regulations" (FDA 1979) and "Handbook for Analytical Control in Water and Wastewater Laboratories" (USEPA 1979). As a minimum, the Quality Assurance program should address the following points:

a. Obtain sample, fill in the required information on a chain-of-custody form. Of critical importance are recording pH of sample as soon as possible (on-site, if practical) and storage in an amber glass bottle with a Teflon-lined cap at 4 C or lower.

b. Maintain sample usage form. Record total amount of sample, where stored and amounts sent to testing laboratories. Before subsample is removed for extraction, the soil must be homogenized to ensure reproducibility of duplicate extracts. Record in raw data when sample was homogenized, date and technician.

c. Dry sample. Record conditions and time needed and amount of Na_2SO_4 used.

d. Perform extraction. Record amount of material, volume of solvent, lot number, supplier, grade of solvent and duration of extraction.

e. Reduce solvent volume and add DMSO. Record amount of DMSO added and final volume of extract. Measure total organic material, if desired.

f. Solvent control for bioassay. Reduce an equivalent amount of solvent and add the same amount of DMSO as needed for the extract. Use equal volumes of negative solvent control in bioassay.

g. Determinations of solvent control and samples spiked with positive controls will be performed as QC checks with each independent bioassay run. The results of those determinations will be recorded in a log book.

h. SOPs for techniques used in the laboratory will be written, and copies of those documents will be kept with the QC log book.

i. As a minimum, one of every ten extractions will be conducted in triplicate to verify the reproducibility of the method.

j. Laboratories conducting these procedures should maintain a Quality Assurance Unit which is responsible for a master schedule, sample receipt log and periodic inspections of the conduct and reporting of each laboratory study.

14.0 Calculations

Total Weight = TW = (soil weight + water weight)

Soil Weight = SW = (TW-WW)

Water Weight = WW = (TW-SW)

$$\text{Moisture Content} = \%W = \frac{(TW-SW)}{TW} \times 100\%$$

Dry Weight of Sample = D = (TW-(SW-%W))

$$\text{Extract is expressed as: } \frac{D}{\text{Volume DMSO}} = \frac{\text{gram equivalents}}{\text{milliliter}}$$

15.0 Precision and Accuracy

Each laboratory using this protocol must determine the accuracy and precision of this method. The precision and accuracy must be determined for both the extraction procedure and the short-term assay of the extracts. A lot of soil will be extracted and determined to be free of mutagenic contaminants. For two spike levels, seven 25-g soil aliquots will be spiked with mutagens or suitable surrogates and the recoveries will be determined. The mean of those recoveries and the relative standard deviation (RSD) of the extractions will be determined and reported. Acceptable mean extractabilities and RSDs will have to be determined based on actual laboratory experiments. In addition to determining the extractabilities of mutagens or surrogates, two levels of both a direct-acting mutagen and a mutagen requiring S-9 activation will be spiked into seven soil aliquots for determination of the mutagenicity of the extracts. The number of revertants at both levels will be compared with the direct determination of those mutagens. The acceptable mean number of revertants and the RSDs of these determinations will be determined on the basis of laboratory work to be performed in validating this method.

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SECTION 7

WASTE SOLIDS

REVIEW OF THE LITERATURE

Introduction

Complex mixtures may be composed of a diverse mixture of organic and inorganic compounds present in a variety of matrices (i.e., solids, liquids and gases). They can be obtained from such widely different sources as the environment or manufacturing and treatment processes. The following document addresses the preparation of complex mixtures from a solid matrix for mutagenicity testing.

Solid Matrix - Definition--

A solid is defined as having relative firmness, coherence of particles or persistence of form as matter that is not liquid or gaseous. In the present document, it will be considered to include tarry materials that are sticky or viscous, such as coal tar sludge and adhesives wastes. It will, however, exclude soil and sediments or solid particulates suspended in a gaseous medium, since these are discussed elsewhere.

The Reason Separation is Necessary--

Many of the complex mixtures are highly toxic to the bioassays. This toxicity in many instances overwhelms the detection of mutagenicity. However, because of possible interactions between components of a complex mixture, care should be taken in interpreting the biological results using fractionated complex mixtures.

Criteria for Sample Preparation--

Various guidelines can be suggested for choosing a sample preparation scheme for use in bioassay evaluation:

a. The selected method should provide a sample that is representative of the original solid matrix sampled within normal limits of biological accuracy and precision.

b. It should not introduce artifacts or change or remove constituents from the complex mixture.

c. The selected methods should be sufficiently tested to have established their validity.

d. The procedure should be simple and cost effective. Methods should be sufficiently rapid to permit repetitive use in the examination of large numbers of samples.

e. As far as possible, the method should minimize the exposure of workers to potential hazards from both the sample itself and the procedure.

f. The methodologies chosen should be compatible with standard analytical techniques.

i. The procedure should remove toxic or growth-stimulating substances which may interfere with the bioassay without altering the overall composition of the sample.

Sources and Sample Collection

The first critical step in preparing a sample for biological analysis is to collect a representative portion of the material to be evaluated. To ensure that the analysis does not over- or underestimate the potential genotoxic effect, representative samples must be obtained. A representative sample contains all constituents in the same ratio present in the bulk material sampled. The probability of achieving this goal is enhanced by compositing multiple samples. These samples can be composited prior to subsampling for extraction or other procedures (USEPA 1982). In some instances, heating or sampling the bulk material at elevated temperatures allows a more homogeneous sample to be collected. However, discretion should be used in utilizing high temperatures, as loss of volatile material may occur. In all instances, mixing should precede sampling where feasible .

The diverse sources of complex solid mixtures and particulates necessitate using a number of different samplers. Table 6 summarizes the recommended samplers for particulates of various sizes and sources of origin (see de Vera et al. 1980).

It is very important that all these samplers be thoroughly cleaned and free from contamination both prior to and between samplings. One simple method to accomplish this is to wash with the solvent to be used. Storage containers should be similarly cleaned. Glass or Teflon should be used as sample containers. The reason for this is that plastic containers are often coated with plasticizers that interfere with biological analysis as well as organic analysis (USEPA 1982).

Extraction

Physical Treatment--

In order to achieve an efficient extraction of a solid matrix, it may be necessary to pretreat the material prior to chemical extraction. If the sample:

a. Contains particles of too large a size - Pretreat the sample by grinding. The necessity for grinding and the choice of apparatus

TABLE 6. SAMPLERS RECOMMENDED FOR VARIOUS TYPES OF SOLID WASTE (USEPA 1982)

Waste type	Waste location or container	Sampling apparatus
Slurries	Tanks, bins, pits, ponds, lagoons	Weighted bottle, dipper
Dry solids	Drums, sacks, piles, trucks, tanks, pits, ponds, lagoons	Thief, scoop, shovel
Sticky or moist solids and sludges	Drums, tanks, trucks, sacks, piles, pits, ponds, lagoons	Trier
Hard or packed wastes	Drums, sacks, trucks	Auger

will depend on the characteristics of the solid matrix. It should be continued until the material can pass through a 1-mm standard sieve or can be extracted through a 1-mm diameter hole (USEPA 1982).

b. Contains volatiles - Subject it to an overnight evaporation at approximately 35 Torr and room temperature in a vacuum manifold (Guerin et al. 1980) or use distillation (Jones et al. 1977) or evaporation (Guerin et al. 1980).

c. Contains liquids - Filter through a Whatman No. 42 filter paper.

Chemical Extraction--

Choice of extraction solvents--Solvents that have been used to extract organic material include n-pentane, benzene, tetrachlorobenzene, methanol, dimethyl ether, methylene chloride, chloroform, carbon tetrachloride, toluene and certain freons (Jobson et al. 1952, Giger et al. 1974, Kincannon 1972, Patel 1974, Raymond et al. 1976, Walker et al. 1975, Sterns et al. 1968). The choice of which solvent should be used can be based on safety, cost, solvent properties and any specific requirements of the extraction procedure. For example, trichlorotrifluoromethane is not very toxic and is slightly superior to carbon tetrachloride in extracting oils when acid and salts are used, and n-pentane does not extract phthalic compounds. However, it should be noted that the USEPA standard for analytical chemical extractions is dichloromethane (USEPA 1982). This solvent can be used to prepare samples of complex mixtures, provided that it is removed before addition to the bioassay.

Extraction methods--

a. Soxhlet extraction method. This method allows for the extraction of semivolatile and nonvolatile organic compounds from solid state materials prior to fractionation. Basically, the method consists of mixing the sample with anhydrous sodium sulfate in an extraction thimble or between two plugs of glass wool and extracting with the appropriate solvent in a Soxhlet extractor.

b. Sonication extraction method. The sonication method produces disruption to ensure intimate contact of the sample matrix with the extracting solvent. It suffers from the disadvantage of producing artifacts and driving off some semivolatile compounds. Basically, the method consists of mixing a ground solid in an extraction medium and then dispersing it using sonication.

c. Blender extraction method. This is perhaps the simplest method of extraction. Basically, it consists of placing a weighed amount of ground sample in a blender together with the extracting solvent and blending. It is a much simpler method than the other two.

Chemical Fractionation--

Chemical fractionation separates the crude extract into a number of groups. The organic constituents of the extract are amenable to evaporation and concentration techniques based on the functional acid-base moieties of the individual complex mixture components. Other methods have been developed for fractionation using chromatography. This separates the complex mixture into individual compound classes (Miller 1982, Boduskznyski et al. 1982a, b, Later et al. 1982, Crowley et al. 1980, Brocco et al. 1973).

Liquid-Liquid extraction--The acid-base liquid-liquid extraction is the simplest and least expensive of all the fractionation schemes. This technique has been used in the chemical and biological analysis of a variety of complex mixtures (Colgrove and Svec 1981), including fossil fuels (Buchanan 1982, Matsushita 1979, Novotny et al. 1981, Pelrov et al. 1981, Epler et al. 1978, Guerin et al. 1980), cigarette smoke condensate (Bell et al. 1969), ground cooked beef (Felton et al. 1981) and environmental samples (Adams et al. 1981, Lundi et al. 1977, Brown et al. 1982, 1983, Grabow et al. 1981). This method is also the basic technique recommended by the USEPA for chemical fractionation for organic chemical analysis (USEPA 1982, Lin et al. 1979).

The acid-base fractionation scheme is based on the acidity constants (pK_as) of organic compounds (see Hendrickson et al. 1970). Compounds with low pK_as are acidic; those with high values are basic. If a complex mixture is equilibrated with an aqueous inorganic acid at low pH (<2), the organic bases should protonate to become water-soluble and positively charged. The insoluble organic acids are thus extractable by solvents.

Conversely, if an inorganic base at a high pH (>12) is used, the organic acids should deprotonate to become water-soluble negatively charged anions; the organic bases are thus extractable by solvents. A complex mixture may be separated into its acid, basic and neutral organic components, together with an aqueous and residual solids fraction, by adjusting the pH of the aqueous phase.

The compounds that are amenable to this type of separation are partially listed in Adams et al. (1983). Either diethyl ether or dichloromethane may be used in the separation procedure, although acetone (Felton et al. 1981) and isooctane (Pelrov et al. 1981) have also been used. Dichloromethane is recommended (USEPA 1982) and can be removed from a separatory funnel without having to remove the aqueous phase because it is denser than water. Ether is more water-soluble, and additional time is required in the extraction procedure. The types of compounds extracted in each fraction include:

a. Organic acids. This class includes varieties of carboxylic acids, guaniacols and phenols (Claeys 1979). These may be identified chemically following chromatographic separation using standard techniques (see USEPA 1982).

b. Organic bases. This fraction includes a variety of nitrogen compounds including alkyl, aromatic and aza-heterocyclic amines. As with the acids, these can be separated further and identified by chromatographic techniques (see USEPA 1982). It is advisable to test this group of compounds as soon as possible, as decomposition and polymerization are likely to occur with time (Worstell and Daniel 1981, Worstell et al. 1981). This may partially account for the variability being detected by some investigators.

c. Neutral organics. A variety of organic compounds are detected in this group. These include aliphatic and aromatic hydrocarbons, as well as oxygenated and chlorinated hydrocarbons. Some workers have included additional separatory steps in their investigation scheme to further delineate this group (Epler et al. 1978, Guerin 1980). These additional separations include using various columns and eluting with different solvents.

d. Water-soluble. This group includes constituents which were not solvent-extractable.

e. Residual solids. Included in this group are inorganics and materials such as coke.

It should be pointed out that some compounds may not survive the acid-base fractionation because of hydrolysis. These include esters, halogenated hydrocarbons, phthalates, nitriles and amides. Some compounds are altered to other compounds. Included in this group are DDT, DDD and toxaphene (USEPA 1980c). In order to reduce the hydrolysis, Felton et al. (1981) used 0.01N acid and base in their extraction procedure, instead of the more commonly used 1N concentration. Still other workers have avoided the difficulty completely by using column-based fractionation schemes (see below). However, Pelrov et al. (1981), in side-by-side studies of liquid-liquid acid-base fractionation and Sephadex LH-20, could detect no mutagenic difference between the two separatory schemes when examining synthetic fuels. Similarly, no difference was detected for extraction of complex mixtures from cooked meats when isopropyl alcohol or acetone was the solvent of choice (Felton et al. 1981). Both of these extraction procedures were 90% efficient, and it was suggested that no artifacts were formed with either extraction method (Felton et al. 1981).

Other schemes of fractionation--These essentially include chromatographic separation using procedures similar to those used to isolate individual compounds (see USEPA 1982). Except for those using Sephadex LH-20 gel and different eluting solvents (Rao et al. 1981, Pelrov et al. 1981, Toste et al. 1982), most of the other separation techniques were applied to specialized situations, such as the analysis of polycyclic aromatic hydrocarbons in marine organisms and sediment (Bjorseth 1979) or the rapid Tenax-GC extraction technique (Shiaras et al. 1980). High-pressure liquid chromatography has also been used directly to determine impurities in carcinogens using the Ames test (Donahue et al. 1978).

Although the standard Ames test or the preincubation modification would be the methods of choice at this time, another method with limited application uses thin-layer chromatography (TLC). This quick and direct method of detecting mutagens by applying the *Salmonella* assay directly onto TLC plates has been used to study mutation associated with photocopier toner and typewriter ribbons (Bjorseth et al. 1982). Others have used TLC plates by extracting the spot (Pitts et al. 1978, Hayashida et al. 1976, Wilson et al. 1980, Issaq et al. 1979). Although this method used mainly pure compounds, it may be useful to screen a large number of complex mixtures. It is an inexpensive test, and response is observed at nanogram amounts. No drawbacks, such as sample dilution or reactions of compounds through the fractionation procedure with irreversible adsorption onto column materials, are thought to occur with this method (similar adsorption could occur on chromatographic columns separating complex mixtures). However, this test is essentially equivalent to the Ames spot test and is thus only a qualitative test. Like the spot test, this method does not detect benzo(a)pyrene or benz(a)anthracene even in the presence of S9 (Bjorseth et al. 1982).

Chemical Isolation--

As mentioned above, various chromatography procedures may be used to individually isolate compounds. The inclusion of these in the initial fractionation scheme may be necessary if the fraction of a particular complexed mixture is toxic in a particular bioassay. It is important when individual mutagenic components are to be identified by chemical analysis. Standard methods for different classes of compounds are available utilizing HPLC, Sephadex LH-20 and silica gel techniques for organic analysis and atomic spectra for inorganic analysis (USEPA 1982).

Concluding Remarks

Many of the fractionation schemes for bioassays have not been verified. In most instances the methods used are similar to those already verified for organic chemical analysis. The inorganic components have not been examined in complex mixtures, although it is known that metals can induce mutation in bacteria (Kanematsu et al. 1980) and carcinogenesis (Saunderman 1978).

The most simple scheme involves blender extraction followed by acid-base liquid-liquid fractionation. Subsequent identification of the individual compounds can be accomplished by separation by various chromatographic means. However, it should be pointed out that as the number of fractions increases, so does the number of bioassays. Thus, more fractions mean more cost. One promising method that seems to alleviate this problem is the direct thin-layer chromatography Ames test. In all instances, irrespective of the fractionation scheme or the bioassay used, the various fractions are usually dissolved in a carrier solvent prior to addition to the bioassay (see Abbondandolo et al. 1980 for discussion of the merits of 13 carrier solvents). Finally, it should also be pointed out that caution should be used in interpreting the result of any bioassay using fractionated solid complex mixtures, as various synergistic and antagonistic mutagenic effects can occur to different extents in the different fractions.

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WASTE SOLIDS WORKGROUP REPORT

Workgroup Tasks

Key Peer Review Comments--

The following points were raised during the initial review. These were listed as an initial starting point in our discussion. It was noted that the purpose of the meeting was to discuss sample preparation for use in bioassays, and not what to do in the bioassay testing. Bioassays were only involved when they affected the method of sample preparation.

1. What is the importance of the normality of the acid and base solutions used in the liquid-liquid extraction?

2. What size should the particles be for the extraction procedure? It has been suggested that 4 mm should be used instead of 1 mm.

3. What are the differences in the three extraction procedures (Soxhlet, blender or sonication)? Is one more efficient and less expensive than another?

4. What storage conditions should be used? Are there any problems or limitations associated with this area of the protocol?

5. What procedure should be used for solvent reduction?

6. Should samples be treated with additional substances (e.g., inclusion of surfactants), and does this enhance the extraction procedure?

7. What do we do about the inorganic chemicals? Where would they occur in an acid-base fractionation scheme?

8. Do we need to include internal standards? Which chemicals should be considered for inclusion? Is this an effective means to evaluate procedures and interlaboratory reproducibility?

9. Are there any special problems associated with a particular group of chemicals (e.g., weak mutagens)?

Issues Identified During the General Sessions and Workgroup Discussion--

Other points were raised during the general sessions. These are listed as a continuation of the points under Key Peer Review Comments so that they can be referred to later.

10. In order to avoid destruction of mutagenic activity, should acid fractionation be conducted prior to base fractionation?

11. Should acid, base and neutral fractions be subfractionated (e.g., weak, strong and precipitated acids)?

12. Which extraction or fractionation procedures have been adequately verified?

13. What are the minimum and maximum number of fractions to be tested in the bioassay?

14. What should be used as upper and lower limits of residue to be tested in bioassay (mg/plate)?

15. What is the scope of this protocol (what types of material should or shouldn't be included)?

16. Should a crude extract using DMSO be tested as an initial sample?

17. How many replicates should be used?

18. What solvents are compatible with the bioassays?

19. What purity of solvents should be used?

20. Redefine waste solids to be compatible with other protocols.

Summary of Workgroup Progress--

All of the above issues were resolved as far as the available scientific data base would allow. In the main, the original protocol was deemed acceptable as a method for preparing samples of the solid matrix for use in bioassay testing. The accomplishments of the workgroup can be summarized as follows:

a. All of the modifications made to this protocol were aimed at reducing degradation of the complexed mixture. The major modifications were:

   - Dissolve the complexed sample in the solvent before addition of the acid or base.
   - Use an acid fractionation before a base fractionation.
   - Conduct all extraction, where possible, at 4 C.

b. An overall scheme of sample preparation and testing was devised.

c. The final definition (Issue 20) was adjusted to be compatible with the definitions for the other types of matrices. This definition was:

A solid waste or solid complexed mixture, obtained from different sources, is defined as that waste or complexed mixture containing less than 50% water and having relative firmness, coherence of particles or persistence of form as matter that is not liquid or gaseous at 25 C. In the present document, it will be considered to include tarry materials that are sticky or viscous (such as coal tar sludge and adhesives wastes), airborne particulates in a gaseous media and solids partitioned from wastewaters or other fluids.

Summary of Key Workgroup Discussions

Major Consensus Opinions--

Conditional statements--

a. It is recognized that liquid-liquid acid-base extraction has the potential of altering components of complexed mixtures and environmental samples. When the objective is an estimation of the overall mutagenicity of the sample, the use of alternative extraction and separation procedures (e.g., HPLC; see Appendix 3) does not appear warranted based on currently available relevant scientific information.

b. Because of the varied nature and sources of the waste solid category, refinement of the existing protocol may be deemed necessary at some future time.

Non-issues (Issues 7 and 17)--In general session, it was generally agreed that analysis of vapor-phase organics and inorganics was not to be considered in the present protocol. Similarly, the number of replicates required will be dictated by the statistical design of the experiment.

Overall extraction and testing scheme (Issues 13, 14 and 18)--To simplify the decision processes involved in the estimation of the mutagenicity of complexed mixtures and environmental samples, the following scheme was devised.

Ideally, the volume of addition to mutagenicity test plates should be held at 50 µL per plate. The dose range considered was 10 ng to 10 mg/plate for nontoxic materials. If a positive nontoxic response is observed in this dose range, no further testing is required. Methods considered for evaluation of toxicity include reduction of the bacterial lawn, zone of growth inhibition in spot tests and calculations of percent survival using an isogenic strain currently being developed for toxicity estimation (Mortelmans K., Mutation Research 1983).

If a toxic response is observed or a negative mutagenicity result is obtained, further fractionation into acid, base and neutral fractions is indicated. In the case of the negative response, such a fractionation is optional and dependent on the experimental design. To minimize the cost of sample preparation for bioassay evaluation, no further fractionation was considered to be warranted unless additional chemical characterization or mutagenicity of individual groups of compounds was required.

Samplers and sampling - pretreatment conditions (Issues 2 and 15)--Adequate documentation already exists on the protocols for sample collection (see USEPA 1982).

Because of the heterogeneous nature of the material included in this category, the following were considered:

a. Reduction in particle size to less than 2 mm was considered adequate.

b. When dealing with oily, gummy or adhesive wastes, additional treatment with anhydrous sodium sulfate or fumed silica gel (Caba-o-sil Corp., Boston, Mass.) may be warranted. Alternatively, size reduction for these materials may be accomplished by the addition of dry ice or by freezing samples and then grinding with dry ice.

c. Liquids will be separated from solids by gravity phase separation for 24 h at 4 C (see Wastewater Protocol).

d. Dust particles in ambient air may also be included in this protocol following the procedures for isolation of airborne particulates discussed in the Air Sample Preparation Protocol.

Storage condition and sample containers--This is an area which needs considerable research to ascertain appropriate conditions. It is recognized that maintenance of the sample in a state which does not alter the integrity of the sample is a necessary prerequisite for success of the protocol. We have determined three areas of storage consideration: (a) original samples of the bulk matrix, (b) crude extracts and (c) processed extract. For practical considerations, storage conditions will be influenced by sample size and number.

When feasible, maintenance of temperatures at 4 C or less should be considered for all samples, and ideally, samples (b) and (c) should be stored at -20 C or less.

As a general rule, preservatives should be excluded.

Samples should be stored in amber glass containers with Teflon caps. In the case of glass-corrosive substances (e.g., hydrofluoric acid) an alternative container made of Teflon is recommended. Stainless steel containers were considered and rejected because of possible contamination of the sample.

Extraction procedures (Issues 3, 5, 6, 16 and 19)--

a. The Soxhlet extraction technique was selected as the principle extraction method, with the possible inclusion of blender techniques as an acceptable alternative procedure. (Initial comparisons of the efficiency of these methods and that of sonication can be found in Adams 1983 and in the section on Soils and Sediments).

Inclusion of additional substances (e.g., surfactants) should be avoided, except where scientific data support their inclusion in order to obtain a greater efficiency of extraction.

b. The solvent selected for extraction was methylene chloride. Dimethyl sulfoxide (DMSO) was not selected for the initial solvation because of possible interferences with chemical analysis and the possibility of a less efficient extraction procedure. The purity of the extracting solvents should be spectroscopic grade or higher.

c. Apparatus for solvent reduction - Although the rotary evaporator may be preferable for particular complexed mixtures, the Kuderna-Danish apparatus described in the protocol will be maintained in order to utilize equipment currently in use in commercial laboratories and to facilitate processing multiple samples.

An aliquot of crude extract will be taken to dryness and redissolved in DMSO at a concentration of 200 mg/mL (or the highest solubility). Although DMSO will be considered as the solvent of choice for bioassay, other solvents, including water, 10% ethanol or acetone, may be used depending on the sample type.

Fractionation procedures (Issues 1, 4, 10 and 11)--

a. Normality of solutions - 1 Normal solutions of acids and bases will be utilized to avoid compound destruction. Bases and acids are added to the sample dissolved in methylene dichloride (or the extracting solvent). The acid separation is also to be undertaken before the base separation to limit the degradation of compounds. Likewise, it is suggested that these separations be undertaken at 4 C.

b. No separation into weak, strong and precipitated (acid, base or neutral) fractions was considered necessary.

c. Only the back-extractable acids, bases and neutral compounds will be evaluated.

d. Hydrochloric acid is preferred to sulfuric acid, as the former causes less decomposition than the latter.

Standardization of procedure (Issue 8)--The only standard on which consensus agreement has been reached is a field blank to ensure that artifacts are not introduced by the extraction procedure.

The selection of additional standards to monitor the extraction procedure and to assess interlaboratory reproducibility was considered; however, the chemical species were not designated. It was recommended that choice of chemicals should be representative with respect to chemical class and extraction properties. The value of the addition of a spike was questioned because of the potential for chemical reactivity with other components of a complex mixture and for possible antagonistic or synergistic responses in a bioassay.

Other Issues--

In addition to the unresolved issues raised in the section on Major Consensus Opinions due to the lack or the limited nature of a scientific data base, various issues of a general nature should be considered:

a. The application of the designated procedure to this category has not been validated when the objective is the preparation of sample for bioassay; however, the use of the protocol for chemical analysis is documented (USEPA 1980).

b. Although vaper-phase and inorganics have been excluded for general consideration, the workgroup has questioned whether the elimination of these chemicals from consideration is appropriate for the waste solid category. Heavy metals are known to exert a mutagenic response in bioassay systems other than the Ames test, and some connections have been established between heavy metals and carcinogenesis. As for the vapor-phase organics, these produce responses in the Tradescantia system and the dessicator assay.

c. Although not directly discussed in the present meeting, it was considered important that other short-term tests, in addition to the Ames system, be used. Several reasons for this are that regulatory decisions are seldom made on the basis of a response in a single system and that additional short-term tests which have the capability of detecting genetic damage other than "mutation" (e.g., chromosome changes, spindle damage and DNA repair capacity) should also be employed. The genotoxic agents may not induce mutation, but may be very effective in inducing aneuploidy. Such a genetic alteration has been linked to cancer and birth defects.

d. Use of chromatographic methodology as an alternative sample fractionation was considered to have numerous disadvantages, including:

- Massive production of solvents
- Expertise required
- Increased cost associated with preparation of sample for mutagenicity testing
- Maximum number of samples per equipment per working day appears to be about eight

However, some members of the general session believed that this method is superior to the liquid-liquid acid-base fractionation scheme.

e. Best approach - With respect to percent chemical recovery, the protocol may not be the most cost effective or practical from the viewpoint of equipment, expertise and the number of samples which can be processed under given time restraints. These questions can be effectively resolved by comparative experimentation with the different methodologies in a cost effectiveness and efficiency study.

Evaluation of Proposed Protocol

The protocol was evaluated in workgroup and in the general session. The opinions of both groups coincided and are summarized below.

Adequacy--

As a first line basis for preparing samples for testing in bioassays, the protocol was considered adequate. However, it was noted that for several crucial areas, such as sample storage or the best method of extraction, a broad data base was unavailable for formulating a scientific decision.

Validity--

One of the main limitations of the waste solid protocol is that the method has only been used for a limited number of samples in a small number of labs. It is assumed that because the methods are, in essence, those used for chemical analysis of organic complex mixtures and environmental samples, they have been well-documented for this type of analysis.

Limitations--

Besides the obvious limitations of the methods to those organics that are back-extractable to the solvent and remain unaltered at the time of assay, one of the limitations of this protocol is the lack of an adequate data base for formulating a specific protocol.

Comparison to Other Protocols--

Similar limitations due to the absence of a data base were also apparent in all but one protocol. The methodologies suggested are comparable to those used for the other media.

Other Data or Information Requirements

Information Gaps--

As indicated above, numerous gaps exist in the scientific data base. These have been divided into major and minor gaps. The major gaps are those questions that are considered necessary to be answered before the protocol can be utilized in a decision making process. Those that are considered minor would improve the efficiency of the protocol and increase the confidence in the data obtained with this process by determining the limitations of each method.

Major gaps--

1. Storage conditions - This is a prerequisite of any of the preparative protocols. Although some data are available on the storage of complex mixtures and environmental samples, no definitive study is available concerning the effect of storage temperature and time on the stability of the samples for analysis with a bioassay system.

2. Validation of the overall protocol - The process for deciding which way to proceed with the extraction and separation procedure was

specifically designed for this protocol. Thus, it is currently untested.

3. Establishment of standardization - Various systems can be proposed to verify the functioning of sample preparation protocols and interlaboratory comparison. However, very few of these have been practically tested.

Minor gaps--

1. Efficiency and limitations of various extraction methods - Only a limited amount of data is available for the most efficient method of extraction. Three areas should be compared: (a) Soxhlet and blender versus sonication, (b) K-D versus rotary evaporator and (c) the efficiency of solvent/solvent mixes.

2. Is the HPLC a viable practical alternative to liquid-liquid acid-base fractionation?

Research Program Needs--

All of the above information gaps are interrelated. The priority of research needs should be directed toward resolution of the major gaps, followed by resolution of the minor gaps.

Resolution of all major gaps could be accomplished in a single series of experiments. Various synthetic mixtures could be evaluated for their stability with respect to a storage time and temperature (e.g., 4, -10, -20 and -80 C). These synthetic mixtures would be composed of combinations of all types of compounds with known variables, such as those compounds that are toxic, nontoxic, mutagenic (both weak and strong), nonmutagenic, acid-extractable, base-extractable, detected in the neutral fraction, direct-acting mutagens or which require activation as well as compounds which are stable and those which are thought to be unstable to liquid-liquid acid-base fractionation schemes. Examination of various synthetic mixes stored at different temperatures and times by the protocol outlined, with parallel chemical characterization, would result in establishment of conditions for storage and provide standards that could be used for interlaboratory comparisons as well as validating the overall protocol.

Similar bioassay response and chemical characterization of known mixtures with composition could then be utilized to compare the efficiency and limitations of the various extraction procedures. A basis for making a rational scientific decision on the use of HPLC as a viable practical alternative to liquid-liquid acid-base fractionation could be made.

The obvious disadvantage to utilizing synthetic mixtures in this type of investigation is the possibility that not all the various permutations will be evaluated.

PROTOCOL FOR THE PREPARATION OF WASTE SOLIDS FOR MUTAGENICITY TESTING

1.0 Scope and Application

Complex mixtures may be composed of a diverse mixture of organic and inorganic compounds present in a variety of matrices (i.e., solids, liquids and gases). They can be obtained from such widely different sources as the environment or manufacturing and treatment processes. The following document presents the best protocols currently available to genetic toxicologists for sample preparation for this matrix.

The methods are limited to those organics that are extractable by the extracting solvent (normally dichloromethane).

2.0 Summary of Method

To simplify the decision process involved in estimating the mutagenicity of complexed mixtures and environmental samples, the following scheme was devised.

Ideally, the volume of addition to mutagenicity test plates should be held at 50 µL per plate. The dose range to be assayed should be 10 ng to 10 mg/plate. If a positive nontoxic response is observed in this dose range with the crude solvent-extract material, no further testing is required.

If a toxic response is observed or a negative mutagenicity result is obtained, further fractionation into acid, base and neutral fractions is indicated. In the case of the negative response, such a fractionation is optional and dependent on the experimental design. To minimize the cost of sample preparation for bioassay evaluation, no further fractionation other than that indicated above is considered to be warranted unless additional chemical characterization or mutagenicity of individual groups of compounds is required.

3.0 Definitions

A solid waste or solid complex mixture, obtained from different sources, is defined as that waste or complex mixture containing less than 50% water and having relative firmness, coherence of particles or persistence of form as matter that is not liquid or gaseous at 25 C. In the present document, it will be considered to include tarry materials that are sticky or viscous (such as coal tar sludge and adhesives wastes), airborne particulates in gaseous media and solids partitioned from wastewaters or other fluids.

4.0 Safety

Ideally, the potential hazards of the solid complex mixture are known. Unfortunately, in many instances this is not the case. Workers should assume that the material is toxic and presents other dangers, such as explosive or flammable tendencies. Each of these hazards should be handled in the appropriate manner. (see Steere 1971, Sax 1965, Bretherick 1975, USEPA 1977, 1980).

5.0 Sample Collection, Preservation and Handling

Equipment and procedures have already been established and described for obtaining solid matrix samples (see de Vera et al. 1980). These are listed in Table 6. The precautions taken for samples to be analyzed for organic analysis are adequate for samples to be prepared for use in the bioassays (see de Vera et al. 1980).

Unless the complex mixtures are known to be unreactive in the presence of light, it is advisable to perform manipulation under reduced light or yellow lighting.

It is recognized that maintenance of the sample in a state which does not alter the integrity of the sample is a necessary prerequisite for success of the protocol. Three areas of storage are to be considered: (a) original samples of the bulk matrix, (b) crude extracts and (c) processed extract. For practical considerations, storage conditions will be influenced by sample size and number.

When feasible, maintenance of temperatures at 4 C or less should be considered for all samples, and ideally, samples (b) and (c) should be stored at -20 C or less.

As a general rule, preservatives should be excluded.

Samples should be stored in amber glass containers with Teflon caps. In the case of glass-corrosive substances (e.g., hydrofluoric acid), an alternative container made of Teflon is recommended.

6.0 Calibration

Blank samples demonstrating that the methods used did not contaminate the sample should be run with each batch of samples prepared.

The number of replicates required will be dictated by the statistical design of the experiment.

7.0 Quality Control

Laboratories preparing samples of waste "solid" should adhere to the Guides for Quality Assurance in Environmental Health Research and the proposed "good laboratory practices" (FDA 1976, 1977, 1978, USEPA 1980). Not only should these practices take place in the laboratory, but they also should be practiced at the point of sampling and transportation. This means that good scientific practices and good administrative record keeping and labeling (see Wastewater Protocol for example of record sheets) should be employed. It is vital that all materials used in the preparation of the sample for bioassay analysis be of spectral grade or equivalent (see de Vera et al. 1980 for further details). The glassware and equipment should also conform to the standards established for cleaning for water and wastewater (de Vera et al. 1980).

8.0 Procedure

8.1 Extraction--

The solid matrix may contain volatiles or be contained in a liquid matrix. Before any material can be used for extraction, the contaminating matrices must be removed. Sufficient material to yield approximately 40 g of solid should be used in each case. This is the raw material to be extracted.

Because of the heterogeneous nature of the material included in this category, the following methods of physical separation may be considered:

a. Reduce particle size to less than 2 mm by grinding.

b. When dealing with oily, gummy or adhesive wastes, additional treatment with anhydrous sodium sulfate or fumed silica gel (Caba-o-sil Corp., Boston, Mass.) may be warranted. Alternatively, size reduction for these materials may be accomplished by the addition of dry ice or by freezing the samples and then grinding with dry ice.

c. Liquids can be separated from solids by gravity phase separation for 24 h at 4 C (see Wastewater Protocol).

d. Dust particles in ambient air are also to be included in this protocol. Follow the procedures for isolation of airborne particulates discussed in the Air Sample Preparation Protocol.

8.2 Soxhlet Extraction Method--

8.2.1 Scope and aplication--This method provides a procedure for extracting organic compounds from a solid matrix prior to fractionation for use in a bioassay.

8.2.2 Summary of the method--The solid sample of <2 mm particulate size is mixed with sufficient sodium sulfate until dry, placed in an extraction thimble or between two plugs of glass wool and extracted using an appropriate solvent in a Soxhlet extractor. The extract is then concentrated and used in the fractionation procedure.

8.2.3 Apparatus and equipment--

a. Soxhlet extractor - 40-mm ID, with 500-mL round-bottom flask.

b. Kuderna-Danish apparatus with a three-ball Snyder column or vacuum rotary evaporator with a chilled solvent receiving flask.

c. Chromatography column - Pyrex, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium. This is used as a drying column.

8.2.4 Reagents and consumable materials--

a. An appropriate solvent or solvent mixture of spectroscopic grade. In most instances, dichloromethane can be used.

b. Anhydrous sodium sulfate, ACS (purified by heating at 400 C for 4 h in a shallow tray).

8.2.5 Procedure--

a. Mix 10 g of the solid with 10 g of anhydrous sodium sulfate and place in a glass extraction thimble. If any problems are encountered, e.g., the sample clogs the thimble, an alternative is to place a plug of glass wool in the extraction chamber, transfer the sample into the chamber and then cover with another plug of glass wool.

b. Place 300 mL of the solvent into a 500-mL round-bottom flask containing a boiling stone (solvent-extracted); attach the flask to the extractor and extract the solids for 16 h.

c. When complete, cool the extract, filter and dry it by passing it through a 4-in column of sodium sulfate which has been washed with the extracting solvent. Collect the dried extract in a 500-mL Kuderna-Danish flask fitted with a 10-mL graduated concentrator tube. Wash the extractor flask and sodium sulfate column with 100 to 125 mL of the extracting solvent. It is then concentrated using the K-D apparatus.

d. Concentration is accomplished by adding one to two solvent-treated boiling chips to the flask and attaching a three-balled Snyder column. Prewet the Snyder column by adding about 1 mL of solvent to the top. Place the K-D apparatus on a steam or hot water bath so that the concentrator tube and the entire lower rounded surface of the flask are bathed in hot water or vapor.

Adjust the vertical position of the equipment and the water temperature to complete the concentration in 15 to 20 min (approximately 45 C). At the proper rate of distillation, the balls of the column will actively chatter, but the chamber will not flood. When the volume of the liquid reaches 1 to 2 mL, remove the K-D apparatus and allow to drain for at least 10 min while cooling.

e. Transfer with solvent washes to a tared sample tube. Dry under nitrogen and weigh residue.

8.3 Blender Extraction Method--

8.3.1 Scope and application--This method provides a procedure for extracting organic compounds from a solid matrix prior to fractionation for use in a bioassay. It is quicker than the standard Soxhlet method. Both methods produce a sample for further fractionation.

8.3.2 Summary of the method--The solid sample of 1 mm particulate size is mixed with sodium sulfate, placed in a blender and extracted using an appropriate solvent. The extract is then concentrated and used in the fractionation procedure.

8.3.3 Apparatus and equipment--

a. Blender with a screw cap (explosion-proof type suggested).

b. Kuderna-Danish apparatus with a three-ball Snyder column or vacuum rotary evaporator with a chilled solvent receiving flask.

c. Chromatography column - Pyrex, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium. This is used as a drying column.

8.3.4 Reagents and consumable materials--

a. An appropriate solvent or solvent mixture of spectroscopic grade. In most instance, dichloromethane can be used.

b. Anhydrous sodium sulfate, ACS (purified by heating at 400 C for 4 h in a shallow tray).

8.3.5 Procedure--

a. Mix 10 g of solid with 10 g of anhydrous sodium sulfate and place in the blender.

b. Add 300 mL of the solvent into the blender. This should half fill the jar. Screw jar cover onto the blender jar tightly.

c. Holding the lid of the blender, blend for 30 sec. Wait 5 min and blend for 15 sec. Repeat the last step.

d. When complete, filter using a Whatmans No. 41 filter paper and dry it by passing it through a 4-in column of sodium sulfate which has been washed with the extracting solvent. Collect the dried extract in a 500-mL K-D flask fitted with a 10-mL graduated concentrator tube. Wash the extractor flask and sodium sulfate column with 100 to 125 mL of the extracting solvent. It is then concentrated using the K-D apparatus.

e. Concentration is accomplished by adding one to two solvent-treated boiling chips to the flask and attaching a three-balled Snyder column. Prewet the Snyder column by adding about 1 mL of solvent to the top. Place the K-D apparatus on a steam or hot water bath so that the concentrator tube and the entire lower rounded surface of the flask are bathed in hot water or vapor. Adjust the vertical position of the equipment and the water temperature to complete the concentration in 15 to 20 min (approximately 45 C). At the proper rate of distillation, the balls of the column will actively chatter, but the chamber will not flood. When the volume of the liquid reaches 1 to 2 mL, remove the K-D apparatus and allow to drain for at least 10 min while cooling.

f. Transfer with solvent washes to a tared sample tube. Dry under nitrogen and weigh residue.

8.4 Liquid-Liquid Extraction-Fractionation--

8.4.1 Scope and application--This method provides a procedure for separating organic compounds from a solid matrix into three main fractions, i.e., acid, base and neutral (aqueous and residual solids are normally discarded). Each of these fractions can be evaluated for mutagenicity in a bioassay.

The method is limited to those organics that are back-extractable to the solvent and remain unaltered at the time of the assay.

8.4.2 Summary of the method--Separation into three major fractions is achieved by a series of liquid-liquid extractions conducted at different pHs and using an appropriate solvent. In some instances, it may be necessary to separate the fractions further (see Section 8.5).

8.4.3 Apparatus and equipment--

a. Separatory funnel with Teflon stopcock (2,000 mL).

b. Kuderna-Danish apparatus with a three-ball Snyder column or a vacuum rotary evaporator with a chilled solvent receiving flask.

c. Drying column - Pyrex, 20-mm ID, approximately 400 mm long, with a coarse fritted plate at the bottom and an appropriate packing medium.

d. Boiling chips, solvent-extracted, approximately 10/40 mesh.

e. Water or steam bath.

8.4.4 Reagents and consumable materials--

a. Sodium hydroxide - (ACS) 1N in distilled water (chilled to 4 C).

b. Hydrochloric acid - (ACS) 1N prepared by mixing concentrated HCl with distilled water (chilled to 4 C).

c. An appropriate solvent or solvent mixture of spectroscopic grade. In most instances, dichloromethane can be used.

d. Anhydrous sodium sulfate, ACS (purified by heating at 400 C for 4 hours in a shallow tray).

e. Distilled water.

8.4.5 Procedure--

a. Dissolve 10 g of the extract in 30 mL of solvent. In most instances, the solvent will be dichloromethane.

b. Place in a separatory funnel.

c. Adjust the pH to 1-2 with HCl (approximately 100 mL). Partition the sample into the two phases by shaking the funnel for one minute with periodic venting to release pressure (point funnel away from the analyst).

d. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete the phase separation. The optimum mechanical technique depends upon the sample, but may include stirring, filtration of the emulsion through glass wool or centrifugation.

e. The organic layer will be the lower phase. This phase will contain the acidic and neutral fraction. Collect the upper phase in a flask and label. This phase contains the base.

f. Reextract the solvent layer twice more with 1N HCl at pH 1-2 (approximately 50 mL each). Combine the aqueous base extracts.

g. Adjust the pH of the aqueous layer obtained in Steps e and f to pH 12-13 by addition of 1N NaOH and extract the base fraction with 200 mL of dichloromethane.

h. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete the phase separation.

i. The organic layer will be the lower phase. This phase will contain the base fraction. Collect the lower phase in a flask and label. This phase contains the base fraction in dichloromethane. Add 100 mL of dichloromethane and repeat the separation to collect a second base fraction in dichloromethane, which is added to the first.

j. Adjust the pH of the solvent layer obtained in Steps e and f to pH 12-13 by addition of 1N NaOH (approximately 200 mL). Partition the sample into the two phases by shaking the funnel for one minute with periodic venting to release pressure (point funnel away from the analyst).

k. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete the phase separation.

l. The organic layer will be the lower phase. This phase will contain neutral fraction. Collect the upper phase in a flask and label. This phase contains the acidic fraction.

m. Reextract the solvent layer twice more with 1N NaOH at pH 12-13 (approximately 50 mL each). Combine the aqueous acidic extracts.

n. The organic layer will contain the neutral fraction.

o. Adjust the pH of the aqueous layer obtained in Steps l and m to pH 12-13 by addition of 1N HCl and extract the acidic fraction with 200 mL of dichloromethane.

p. Allow the fractions to separate in an upright stand (10-min minimum). If the emulsion interface between layers is more than one-third the size of the solvent layer, the analyst must employ a mechanical device to complete the phase separation.

q. The organic layer will be the lower phase. This phase will contain the acidic fraction. Collect the lower phase in a flask and label. This phase contains the acid fraction in dichloromethane. Add 100 mL of dichloromethane and repeat the separation to collect a second acidic fraction in dichloromethane, which is added to the first.

r. The dichloromethane fractions obtained in Step i (base fraction), Step n (neutral fraction) and Step q (acid fraction) are concentrated separately. The corresponding aqueous fractions and any precipitate remaining represent the other two fractions (these are normally discarded).

s. Concentration is accomplished by adding one to two solvent-treated boiling chips to the flask and attaching a three-balled Snyder column. Prewet the Snyder column by adding about 1 mL of solvent to the top. Place the K-D apparatus on a steam or hot water bath so that the concentrator tube and the entire lower rounded surface of the flask are bathed in hot water or vapor. Adjust the vertical position of the equipment and the water temperature to complete the concentration in 15 to 20 min (approximately 45 C). At the proper rate of distillation, the balls of the column will actively chatter, but the chamber will not flood. When the volume of the liquid reaches 1 to 2 mL, remove the K-D apparatus and allow to drain for at least 10 min while cooling.

t. Transfer with solvent washes to a tared sample tube. Dry under nitrogen and weigh residue for the base, acid and neutral fractions. These fractions can be tested in the bioassay.

8.5 Chemical Isolation--

This step is only necessary if the fractionation scheme giving base, acid, and neutral fractions is insufficient to reduce the toxicity to a level where the mutagenicity can be examined by the bioassay system or if the identity of the individual compounds is required. If this is the case, standard procedures are already established for chemical analysis that are compatible with the biological systems (see EPA 1982a).

9.0 Calculations

The type of calculations used will depend upon the source of the sample and the experimental design. For instance, the original bulk sample could be separated into different matrices, each of which follows a separate extraction-fractionation scheme. The samples obtained from each of these schemes could then be composited (e.g., crude extract from a liquid matrix combined with the crude extract from a solid matrix) or treated separately for analysis by the bioassay system.

Irrespective of the experimental design, for each stage of the extraction-fractionation scheme, the dry weight of each component should be recorded (e.g., weight of original bulk sample, crude extract, acidic fraction and neutral fraction). The genetic event for each bioassay can then be calculated using standard methods accepted for each assay (see Williams and Preston 1983 for details for the Ames test). It is suggested that the response of each bioassay be expressed as genetic event/mg weight (e.g., revertants/plate/mg for the Ames system) of the sample fraction under consideration.

10.0 Precision and Accuracy

Many of the fractionation schemes for bioassays have not been verified. In most instances, the methods used are similar to those already verified for organic chemical analysis. The most simple scheme involves blender extraction followed by base-acid liquid-liquid fractionation. Subsequent identification of the individual compounds can be accomplished by separation by various chromatographic means. However, it should be pointed out that as the number of fractions increases, so does the number of bioassays. Thus, more fractions mean more cost.

Finally, it should also be pointed out that caution should be used in interpreting the result of any bioassay using fractionated solid complex mixtures, as various synergistic and antagonistic mutagenic effects occur to different extents in the different fractions.

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APPENDIX 1

TR-506-105B

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continued-

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APPENDIX 2

TR-506-106

AGENDA

MUTAGENICITY SAMPLE PREPARATION PROTOCOLS

PANEL MEETING

July 23-25, 1984
Hyatt Rickeys
Palo Alto, California

EPA Task Manager: Dr. Llewellyn Williams

Time	Agenda Item[a]	Individual
Monday, July 23, 1984		
8:00 a.m.	Welcome	J. Heaton
	Administrative Announcements	J. Heaton
8:15 a.m.	Panel Meeting Overview	L. Williams
	1. Background	
	2. Objectives	
	3. Assumptions	
	4. Approach and Schedule	
	5. Introduction of Workgroup Leaders	
	Presentations of Sample Preparation Protocols	
	1. Summary of Air Protocols	
9:00 a.m.	• Review paper	V. Ramanujam
9:20 a.m.	• Group discussion/recommendations	L. Williams
9:50 a.m.	• Summary of group discussion and workgroup tasks	V. Ramanujam

continued-

(a) Panel meeting observers are welcome to participate in all discussion periods and workgroup meetings. Degree of observer participation in workgroup meetings to be determined by Workgroup Leader.

Appendix 2 - continued

Time	Agenda Item	Individual
Monday - continued		
	2. Summary of Drinking Water Protocols	
10:00 a.m.	• Review paper	W. Tabor
10:20 a.m.	• Group discussion/recommendations	L. Williams
10:50 a.m.	• Summary of group discussion and workgroup tasks	W. Tabor
	3. Summary of Wastewater Protocols	
11:00 a.m.	• Review paper	D. Brusick
11:20 a.m.	• Group discussion/recommendations	L. Williams
11:50 a.m.	• Summary of group discussion and workgroup tasks	D. Brusick
12:00 Noon	Lunch	
	4. Summary of Nonaqueous Liquid Wastes Protocols	
1:30 p.m.	• Review paper	R. Spanggord
1:50 p.m.	• Group discussion/recommendations	L. Williams
2:20 p.m.	• Summary of group dicussion and workgroup tasks	R. Spanggord
	5. Summary of Soils and Sediments Protocols	
2:30 p.m.	• Review paper	K. Brown
2:50 p.m.	• Group discussion/recommendations	L. Williams
3:20 p.m.	• Summary of group discussion and workgroup tasks	K. Brown
	6. Summary of Waste Solids Protocols	
3:30 p.m.	• Review paper	B. Scott
3:50 p.m.	• Group discussion/recommendations	L. Williams
4:20 p.m.	• Summary of group discussion and workgroup tasks	B. Scott
4:30 p.m.	Initial Workgroup Meetings	Workgroup Members
5:00 p.m.	Adjourn for Day	

continued-

Appendix 2 - continued

Time	Agenda Item	Individual
Tuesday, July 24, 1984		
8:00 a.m.	Introductory Remarks	L. Williams
8:15 a.m.	Workgroup Meetings 1. List workgroup tasks 2. Begin revising protocols per group discussion 3. List unresolved issues	Workgroup Members
	Workgroup Status Reports ● Status of workgroup tasks ● Unresolved issues ● Group discussion ● Revised tasks for workgroup	
10:30 a.m.	1. Status of Air Workgroup	V. Ramanujam
10:45 a.m.	2. Status of Drinking Water Workgroup	W. Tabor
11:00 a.m.	3. Status of Wastewater Workgroup	D. Brusick
11:15 a.m.	4. Status of Nonaqueous Liquid Wastes Workgroup	Y. Wang
11:30 a.m.	5. Status of Soils and Sediments Workgroup	K. Brown
11:45 a.m.	6. Status of Waste Solids Workgroup	B. Scott
12:00 Noon	Lunch	
1:30 p.m.	Workgroup Meetings 1. List revised workgroup tasks 2. Continue revising protocols per group discussion 3. Resolve issues	Workgroup Members
	Workgroup Status Reports ● Status of workgroup tasks ● Unresolved issues ● Group discussion ● Revised tasks for workgroup	
3:30 p.m.	1. Status of Air Workgroup	V. Ramanujam
3:45 p.m.	2. Status of Drinking Water Workgroup	W. Tabor

continued-

Time	Agenda Item	Individual
Tuesday - continued		
4:00 p.m.	3. Status of Wastewater Workgroup	D. Brusick
4:15 p.m.	4. Status of Nonaqueous Liquid Wastes Workgroup	Y. Wang
4:30 p.m.	5. Status of Soils and Sediments Workgroup	K. Brown
4:45 p.m.	6. Status of Waste Solids Workgroup	B. Scott
5:00 p.m.	Adjourn for Day(a)	L. Williams
Wednesday, July 25, 1984		
8:00 a.m.	Introductory Remarks	L. Williams
8:15 a.m.	Workgroup Meetings 1. List workgroup tasks 2. Continue revising protocols per group discussion 3. Resolve issues and summarize	Workgroup Members
	Workgroup Summary Presentations • Summary of workgroup consensus • Group discussion • Final recommendations	
10:30 a.m.	1. Summary of Air Protocol	V. Ramanujam
11:00 a.m.	2. Summary of Drinking Water Protocol	W. Tabor
11:30 a.m.	3. Summary of Wastewater Protocol	D. Brusick
12:00 Noon	Lunch	

continued-

(a) Meeting room available for informal work group meetings in the evening to achieve consensus on unresolved issues.

Appendix 2 - continued

Time	Agenda Item	Individual
Wednesday - continued		
	Workgroup Summary Presentations (continued)	
1:30 p.m.	4. Summary of Nonaqueous Liquid Wastes Protocol	Y. Wang
2:00 p.m.	5. Summary of Soils and Sediments Protocol	K. Brown
2:30 p.m.	6. Summary of Waste Solids Protocol	B. Scott
3:00 p.m.	Preparation of Draft Workgroup Reports and Revised Protocols	Workgroup Members
4:40 p.m.	Call for Draft Workgroup Reports and Revised Protocols	J. Heaton
4:50 p.m.	Closing Remarks	L. Williams
5:00 p.m.	Adjourn	

APPENDIX 3

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROTOCOL

REVIEW OF LITERATURE

Introduction

Residue organics isolated from numerous environmental, waste and biological samples have been tested for mutagenicity and other biological end points via a variety of short-term bioassay procedures (Hoffman 1982). These studies have pursued three lines of investigation: (1) development of short-term bioassays to assess the mutagenicity/carcinogenicity of the residue organics; (2) development of isolation methods to prepare samples of residue organics from environmental, waste and biological matrices that are suitable for bioassay and (3) development of fractionation methods to isolate individual mutagens/carcinogens from the complex mixture of residue organics for further biological assessment or for compound identification. Progress in the area of short-term tests of individual chemicals and of mixtures of residue organics has been noteworthy over the past decade. This progress has been documented in numerous publications, including published reports of a biannual symposium on short-term testing of complex mixtures (Waters et al. 1978, 1980, 1983, 1984), of guidelines and recommendations of the use of short-term tests (e.g., the USEPA Gene-Tox Program and the program of the International Commission for Protection Against Environmental Mutagens and Carcinogens, both of which are periodically detailed and updated in the journal, Mutation Research), of guidelines for the assessment of toxicity and biohazards of environmental mixtures (e.g., Level 1 Biological Testing Program, USEPA 1981, 1983) and of an interim quality assurance program for the use of the Salmonella mutagenicity test in the bioassay of chemicals and mixtures of residue organics (Williams and Preston 1983). However, progress in the area of sample preparation has been less dramatic. The protocols detailed in this document address the gaps of standardization and recommendations of procedures for the isolation of residue organics from environmental and waste samples for mutagenicity testing. One problem associated with each of these protocols is the lack of approaches to the fractionation of the complex mixtures of residue organics. Such separation methods are necessary, not only for the aforementioned reasons, i.e., compound isolation for chemical identification and further biological testing, but also to address problems associated with the initial bioassays of the residue organics prepared via the protocols. At least two problems are apparent: (1) results of the initial bioassay of the residue organics showing toxicity instead of a dose response and (2) results of the initial bioassay showing no response. Both of these results could be due to the presence of antagonistic and/or toxic components present in the original mixtures of residue organics. Therefore, an initial fractionation of this mixture and subsequent bioassay of the subfractions would allow for a more accurate assessment of the mutagenicity of the residue organics.

Progress in the fractionation of complex mixtures of residue organics for bioassay of components has been limited. In general, three types of fractionation procedures have been investigated for use in the separation of mixtures of residue organics from environmental and waste samples. These are: (1) extraction methods, including both liquid-liquid and liquid-solid techniques; (2) thin-layer chromatography (TLC) methods and (3) high-performance liquid chromatography (HPLC) methods, including both normal phase and reverse phase separation. The methods were developed for studies of the potential biohazards associated with specific media, e.g., drinking water or air particulates, or with general contamination questions involving several media, e.g., synthetic fuel technologies and hazardous waste sites. The specific focus of some of these studies has been to isolate the mutagenic components from a particular complex mixture of residue organics for additional biological assessment and for compound identification. The salient features of the use of these methods, with particular attention to HPLC techniques, are discussed in the following sections.

Overview of Fractionation Methods to Separate Complex Mixtures of Residue Organics for Bioassay

Any method for the fractionation of residue organics for subsequent bioassay of subfractions should be reasonably quantitative and free of artifacts of isolation. The desired strategy would apply reliable and reproducible methods which result in the rapid separation of complex mixtures containing a wide range of classes of organic compounds. Since the complex mixtures of residue organics isolated from environmental and waste samples via the protocols described in this document are composed of a vast number of unknown compounds, many of the reported fractionation methods, although sample specific, would not be applicable to the diversity of residue organics under consideration. Therefore, the methodology to be employed for the fractionation of these residue organics should have wide applicability. As summarized in the following sections, it appears that HPLC fractionation is applicable to a wide variety of sample types in addition to closely meeting the desired criteria described above.

Fractionation Studies of Residue Organics Isolated from Air--

The collection of air samples, described in this document involves the collection of two sample types, both particulate and vapor phase organics. Biological testing and fractionation studies of air samples from automobile and coal combustion emissions were the subject of a symposium reported by Holmberg and Ahborg (1983). Numerous other reports and reviews have appeared (e.g., Claxton 1983, Epler 1980). One general approach to the fractionation of these samples was reported by Eisenberg (1978) in which HPLC was used for class fractionating the organic material extracted from atmospheric particles. Initial fractionation was by normal phase HPLC, and further fractionation of the polynuclear aromatic hydrocarbons was by reverse phase HPLC.

Subsequent studies by Schuetzle et al. (1981) led to the isolation via HPLC of mutagenic components of the soluble organic fraction of particulate matter from diesel exhausts. One important point of this extensive study was that the HPLC procedure gave >95% recoveries of both the mass and mutagenic activity of the sample extracts of residue organics. Other noteworthy studies of the residue organics from air samples via HPLC include: Those of Leary et al. (1983), in which phenalen-1-one was identified as a toxicologically significant component in fossil fuel combustion emission; those of Tokiwa et al. (1983), in which mutagenic nitro compounds were found on exposure of nonmutagenic aromatic/heterocyclic constituents of airborne particulates to nitrogen dioxide; those of Harris et al. (1984), in which nitropyrene was shown to be a contributing mutagenic factor in coal fly ash particles and those of Alfheim (Alfheim and Ramdahl 1984, Alfheim et al. 1984), in which wood combustion emission extracts of residue organics were shown initially to be toxic on mutagenicity testing, and HPLC fractionation led to a more accurate assessment of the mutagenic potential of these samples. The conclusion drawn from these representative studies of residue organics isolated from air samples is that HPLC can be effectively utilized to separate extracts for a more accurate determination of the mutagenicity associated with the organic constituents.

Fractionation Studies of Residue Organics Isolated from Drinking Water and Wastewaters--

Samples of residue organics from both drinking water and the aqueous portions of wastewater are prepared according to similar protocols, as described in this document. Therefore, fractionation studies of the residue organics from both sample types for mutagenicity testing will be considered together in this section. The solids portions (i.e., sludges) of the wastewater samples will be discussed in the section on Soils and Sediments.

The need for the fractionation of residue organics isolated from water samples to assess mutagenicity was presented in an overview of short-term biological tests by Loper (1980a). The toxicity and nonlinear dose-response curves obtained on initial bioassy of such residue organics appeared to be commonplace for these samples. Therefore, sample fractionation is warranted to more accurately assess the mutagenicity of these samples.

The use of HPLC techniques to separate residue organics from wastewater samples for further characterization was introduced by Jolley et al. (1975). Crathorne et al. (1979) proposed an HPLC approach to the identification of organic constituents in the residue organics of drinking water. At this time, Jolley and Cumming (1979) separated complex mixtures of nonvolatile organics in polluted waters by HPLC for bioscreening. A more general HPLC approach was introduced by Tabor (Tabor et al. 1980, Tabor and Loper 1980) for the fractionation of residue organics from drinking water that featured assessment of the distribution of residue organics by polarity using an analytical HPLC separation followed by preparative scale HPLC separation on the residue organics for mutagenicity testing. This approach led to the first identification of a previously unidentified mutagen from drinking water residue organics (Tabor 1983).

The use of HPLC for the separation of residue organics from aqueous samples for mutagenicity testing has been extended to the studies of many other types of water samples from different parts of the world. Baird and co-workers (Baird et al. 1980, Nellor et al. 1984) have utilized HPLC in their studies of the mutagenicity of residue organics from drinking water, river water, storm runoff and reclaimed wastewaters and other wastewaters. Cumming et al. (1980) have applied HPLC to the separation of wastewater effluent residue organics to study the relationship of mutagenicity to disinfection methods. In a study of genotoxic components of oil shale retort process water, Strniste et al. (1983) first separated these samples by a classical liquid-liquid extraction scheme followed by HPLC separation of the extracts for bioassays. One Dutch research group (Kool et al. 1984) has reported the use of HPLC to fractionate drinking water residue organics for mutagenicity assessment.

The most extensive use of HPLC for the fractionation of drinking water and wastewater residue organics has been by Tabor and Loper (citations above, Loper et al. 1983, 1984, Tabor and Loper 1984a, Tabor et al. 1984). In these studies, residue organics from a variety of waters, i.e., ground, river, effluent, drinking, etc., have been fractionated. In some cases, toxicity was observed for the isolated residue organics (e.g., Loper et al. 1983, Tabor and Loper 1984a) removed from the mutagenic constituents on HPLC fractionation. This approach has provided for a better assessment of the mutagenic potential of such residue organics. Further discussion of this methodology is presented in the following sections.

Fractionation Studies of Residue Organics Isolated from Soils and Sediments--

The isolation of residue organics from soils and sediments generally has followed classical extraction methodologies, as described in the Protocol. The application of column chromatography for the fractionation of such samples has been limited. For example, Donnelly et al. (1983) have reported the use of normal phase chromatography in the fractionation of residue organics from soils and hazardous waste-amended soils for mutagenicity testing. Both normal phase and reverse phase HPLC have been utilized by Tabor and co-workers (Tabor et al. 1984, Tabor et al. in preparation) in studies of the mutagenicity of residue organics isolated from sludges obtained from municipal sewage treatment plants heavily impacted by industrial influents. Although HPLC has had limited application for samples prepared from these media, preliminary studies suggest value in the application of this technique.

Fractionation Studies of Residue Organics Isolated from Nonaqueous Liquids and from Waste Solids--

These media were combined for discussion, since the protocols for the isolation of residue organics are similar and the application of HPLC fractionation of these samples is similar. Numerous applications of HPLC to synthetic fuels, oils, etc., have been reported to prepare fractions for bioassay. Fan et al. (1977) reported the use of HPLC in isolating N-nitrosodiethanolamine from cutting fluids for bioassay, whereas Goekner et al. (1977) reported the isolation of methyl chrysenes from a coal liquefaction product. In addition to the application of chromatographic techniques to these sample types, the research group at the Oak Ridge National Laboratory (e.g., Jones et al. 1977, Rao et al. 1981) have reported the use of HPLC techniques for the separation of residue organics from synthetic fuel technologies for bioassays. Toste et al. (1982) reported that HPLC fractionation facilitated the bioassays of synfuels. Also, chromatographic fractionations have been reported by Haugen et al. (1982) and Benson et al. (1982) for the isolation of fractions of biohazardous substances in other synthetic fuel products.

The application of HPLC to the separation of residue organics from solid wastes for mutagenicity testing has been limited. Donahue et al. (1978) reported the detection of mutagenic impurities in HPLC subfractions of carcinogens and noncarcinogens. Donnelly et al. (1983) reported the normal phase chromatographic fractionation of hazardous wastes for the preparation of fractions for a variety of short-term bioassays.

Fractionation Studies of Residue Organics from Biological Samples for Mutagenicity Studies--

In addition to residue organics isolated from the six media discussed in this document, residue organics have been isolated for a variety of biological samples and subsequently fractionated via HPLC for mutagenicity testing. A few examples of these studies are warranted. Putzrath and co-workers (Putzrath et al. 1981, Putzrath and Eisenstadt 1983) have reported the isolation of residue organics from urine for subsequent fractionation via reverse phase HPLC for the isolation of fractions for mutagenicity testing. Similar studies have been reported by Marshall et al. (1983). Residue organics extracted from human feces have been fractionated by HPLC techniques for mutagenicity assessment by Reddy et al. (1984) and by Dion and Bruce (1983). Extracts of AAtex-treated corn were fractionated by reverse phase HPLC for mutagenicity testing (Singh et al. 1982). These are but a few of the examples of the use of HPLC in other areas for the fractionation of residue organics to conduct mutagenicity tests.

Use of HPLC for the Fractionation of Residue Organics

As can be seen, HPLC has been applied successfully to a wide variety of samples of residue organics for the preparation of residue organics for mutagenicity testing. A few comments on the use of the technique and possible limitations are warranted.

Development and Use of HPLC--

The development of the protocol recommended for the HPLC separation of residue organics for subsequent mutagenicity testing was based upon a number of investigations. These have included applicability to a variety of samples, choice of columns and mobile phases and subsequent preparation of the HPLC subfractions for bioassays. As previously noted, HPLC has been applied to a wide variety of samples of residue organics.

To separate these residue organics, the choice of HPLC columns has been an outgrowth of the nature of the separation question. The most widely used type of HPLC column for the separation of residue organics has been the reverse phase column, i.e., a silica gel modified to an octadecylsilane (see Tabor 1984 for a discussion of the types and mechanisms of HPLC separations). In a reverse phase HPLC separation, the more polar constituents in a sample are eluted first, followed by the elution of increasingly nonpolar constituents. It is with this latter group of organic compounds, i.e., the nonpolar constituents, that reverse phase has the widest application to the separation of complex mixtures of residue organics from environmental and waste samples (e.g., Hoffman 1982, Loper 1980b, literature cited in preceding sections). The second most widely used type of HPLC column for the separation of residue organics has been the normal phase column, i.e., silica gel (see Tabor 1984). In a normal phase HPLC separation, the nonpolar constituents in a sample are eluted early, followed by the elution of increasingly polar constituents. Normal phase HPLC has been widely used for the separation of nonaqueous liquids for the preparation of subfractions for bioassays. For example, Fan et al. (1977) isolated the semipolar N-nitrosodiethanolamine from cutting fluids via silica chromatography. Also, normal phase HPLC has been used for the fractionation of some air sample residue organics (e.g., Alfheim et al. 1984). Other applications of normal phase HPLC to the separation of residue organics for mutagenicity testing care are contained in the citations of the previous sections.

In addition to the suitability of both reverse phase and normal phase HPLC to the separation of constituents of residue organics from environmental and waste sample, the technique is not only highly reproducible and reliable, but also gives good recoveries of residue organics in terms of mass and mutagenicity (e.g., Schuetzle et al. 1981, Loper and Tabor 1983, Tabor and Loper 1984b). This is important when mass balances and accountability of mutagenicity of the sample constituents are important criteria for a study. Such is the case in types of anticipated use of the protocols described in this document. For example, TLC separations do not give quantitative or even good recoveries of mutagenic components separated from complex mixtures of residue organics (see Tabor et al. 1980 for examples of several types of TLC separation). Likewise, many liquid-liquid and liquid-solid extraction schemes show evidence of altering not only the chemical composition of the samples of residue organics but also the amount of mutagenesis of the sample (see Tabor et al. 1984). From the results of the studies cited in the preceding sections, HPLC appears to be superior to TLC and extraction in terms of recovery and sample alterations.

Another question is in order regarding the use of HPLC for the separation of residue organics for mutagenicity testing. This is the choice of solvents both in terms of sample compatibility and HPLC system compatibility. The solvents should not react with sample constituents to produce artifacts of isolation. For example, diethyl ether and similar ethers readily form peroxides, which are reactive with many organic functional groups (e.g., alcohols, phenols). Many solvents contain impurities which may react with sample constituents or may be mutagenic themselves (Tabor and Loper, unpublished). Therefore, not only must high quality solvents be used, but also a solvent blank should be run with each sample set and processed for mutagenicity testing. The procedure is described in the attendant Protocol. In terms of HPLC system compatibility, the solvents used in any one separation should be miscible, and the solvents should be compatible with the HPLC detector (e.g., nonabsorbing at the wavelength used for UV detection).

Solvents used for HPLC separation should be easily removed from the HPLC subfractions so that the separated components can be tested for mutagenicity. For example, Maron et al. (1981) reported that not all solvents are compatible with the Salmonella mutagenicity test, whereas Anderson and McGregor (1980) reported that although some solvents are compatible with the Salmonella mutagenicity test, the use of different solvents with comparable samples gave different yields of revertants. Therefore, HPLC subfractions should be prepared in comparable ways for mutagenicity testing. One common method for solvent removal is via gentle evaporation (e.g., Baird et al. 1980, Nellor et al. 1984, Loper and Tabor 1983, Loper et al. 1984). In this case, the subfraction is evaporated to dryness and redissolved into dimethyl sulfoxide (DMSO) for mutagenesis testing. A description of this evaporative procedure is detailed in the Protocol section. A second method for preparing HPLC subfractions for mutagenicity testing has been described by Tabor and co-workers (Tabor et al. 1980, Tabor and Loper 1980). In particular, this method was developed to remove water/acetonitrile solvent mixtures from HPLC subfractions isolated via reverse phase chromatography. The isolated HPLC subfractions are diluted with two to three volumes of water, and this dilute solution is passed through a sample enrichment and purification cartridge (SEP-PAK[a]) containing octadecylsilane. Following removal of the last portion of solvent from the SEP-PAK the HPLC subfraction residue organics are eluted with a small volume of methylene chloride. The eluate is then concentrated via evaporation for mutagenicity testing. This procedure is detailed further in the protocol section.

Recommendations and Limitations--

The goal of this fractionation procedure is to prepare subfractions or residue organics suitable for mutagenicity testing. This is necessitated by the fact that some residue organics isolated from environmental and waste samples may show either toxicity on initial mutagenicity testing or no dose response on initial testing. Therefore, fractionation would be utilized to separate the sample into broad groups of organic constituents, ranging from polar to nonpolar, for mutagenicity testing. The reports of Baird and co-workers (Baird et al. 1980, Nellor et al. 1984), Alfheim and co-workers (Alfheim et al. 1984, Alfheim and Randahl 1984) and Tabor and co-workers (Tabor and Loper 1980, 1984a, 1984b, Tabor et al. 1980, 1984, Loper and Tabor 1983, Loper et al. 1983, 1984) have shown that HPLC is the method of choice for the stated goal of this fractionation procedure. The method features an analytical scale reverse phase (or normal phase where warranted) HPLC separation of an aliquot of the residue organics to assess the distribution of organic constituents in the sample according to polarity. The bulk of the sample is separated via preparative scale HPLC, and the subfractions are processed for bioassay. The criteria for using reverse phase or normal phase are described in the Protocol section. The method has been shown to be widely applicable and to give reproducible, quantitative results without artifacts of isolation (citations above). The method also can be used as a starting point for the isolation of mutagens from residue, organics for compound identification and chemical/biological characterization.

There are several limitations to the method. For application to mixtures of residue organics containing salts or ionic substances, the general approach described in the Protocol is applicable, but columns containing different

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(a) Registered trademark.

chromatographic stationary phases may be required. This area needs further research, and each sample will have to be evaluated on a case-by-case basis for suitability. A second limitation should be mentioned, although it is not serious. The successful use of the HPLC protocol for the separation of residue organics will require some background and training, but does not require a specific degree in analytical chemistry. Many of the HPLC instrumentation companies offer training courses both in the field and at specific locations. Recently, an overview on chromatography (Tabor 1984) was published to introduce the laboratory technician to the theory and principles of chromatography. Additionally, this overview contains numerous references to specific books, reviews and technical articles on HPLC that may be of interest to the user of this Protocol.

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PROTOCOL FOR THE SEPARATION OF RESIDUE ORGANICS FOR MUTAGENICITY TESTING

1.0 Scope and Application

1.1 This method is to be used for the separation of residue organics isolated from environmental matrices for subsequent mutagenesis bioassay of the separated organics.

1.2 The method is suggested for residue organics that show toxicity or no dose response on initial mutagenesis bioassay. The method is applicable to residue organics isolated from environmental and waste samples via methods described in this protocol document.

1.3 The method can be used as a starting point for the isolation of mutagens from residue organics for compound identification and chemical/ biological characterization.

1.4 The method is applicable to mixtures of residue organics containing mostly semipolar and nonpolar constituents. For application to mixtures of residue organics containing salts or ionic substances, the general approach is applicable, but different chromatographic stationary phases may be required. Further research on a case-by-case basis may be required.

1.5 This method is restricted to use by or under the supervision of analysts experienced in chromatography and properly trained in the handling and use of biohazardous materials.

2.0 Summary of Methods

2.1 Residue organics showing toxicity or no dose response on initial mutagenesis assessment are fractionated via HPLC, and the isolated subfractions are tested for mutagenicity. The separation process involves a minimum of two steps: an initial analytical scale HPLC separation of an aliquot of the sample to assess the distribution of constituents in the sample according to their polarity and a preparative scale HPLC separation of the sample, wherein fractions are collected for mutagenesis bioassay.

2.2 The method described in this protocol is based on reports by Tabor and Loper (1980, 1984a, b), Tabor et al. (1980, 1984), Loper and Tabor (1983), Loper et al. (1983, 1984), Baird et al. (1980), Nellor et al. (1984) and Alfheim et al. (1984) for the fractionation of complex mixtures of residue organics isolated from environmental and waste samples. The design and application of the method are described in the above list of references. The method has been successfully used in the separation of residue organics exhibiting toxicity on initial bioassay. Additionally, separated fractions have indicated the presence of synergistic and antagonistic constituents of the original mixture of residue organics.

3.0 Definitions

3.1 ASTM Type I Water - American Society for Testing Materials (ASTM) defines Type I water as having a maxiumum total matter of 0.1 mg/L, a maximum electrical conductivity at 25 C of 0.06 μmho/cm, a minimum electrical resistivity at 25 C of 16.67 Mohm·cm and a minimum color retention time for potassium permanganate of 60 min.

3.2 HPLC - High Performance Liquid Chromatography

3.3 Residue Organics - Complex mixtures of organics, isolated from environmental and waste samples via protocols described in this document for mutagenesis testing.

3.4 Solvent Blank - Elution solvents are concentrated 1,000 times and the concentrates are bioassayed for mutagenesis.

3.5 System Blanks - HPLC separations of injected sample solvents, separated according to the method utilized for the sample of residue organics.

4.0 Interferences

4.1 Interferences to the mutagenicity tests from elution solvent will vary from supplier to supplier and from grade to grade. Therefore, it is recommended that pesticide grade or equivalent solvents be used and that appropriate tests of each lot of solvent be conducted according to the procedures described in Section 9.1.

4.2 Interferences to mutagenicity tests and subsequent chemical analyses from the HPLC apparatus and glassware can be avoided by using only equipment containing stainless steel, glass and TFE components. Samples and solvents should come in contact only with glassware which has been properly cleaned, as described in Section 6.2.

5.0 Safety

The toxicity or carcinogenicity of residue organics separated in this method have not been defined, however, each sample and subsequent HPLC fractions should be treated as a potential health hazard. Procedures for handling such materials have been described (USNCI 1981).

6.0 Apparatus and Materials

6.1 HPLC Unit--

6.1.1 An HPLC binary gradient system consisting of two pumps (Waters Associates Model 510 or 590 or equivalent), a gradient controller capable of generating combinations of isocratic and gradient solvent elutions during sample separation (Waters Associates Model 680 or equivalent), a five position automatic solvent switching valve (Waters Associates Autochrome(a) 101 or equivalent), and a variable volume, to 2.0 mL, loop injector (Waters Associated Model U6K or equivalent). All connecting tubing should be of stainless steel and TFE composition.

6.1.2 A radial compression model HPLC column unit (Waters Associates RCM-100(a) Number 84800 or Waters Associates Z-Module(a) Number 86500 or equivalent) fitted with 8 mm by 10 cm Radial-PAK(a) cartridge packed with 10 µ octadecylsilane (Waters Associates number 84770 or equivalent) for reverse phase separation or packed with 10 µ silica (Waters Associates number 84730 or equivalent) for normal phase separations. Alternatively, other types of preparative scale HPLC columns, reverse phase and normal phase, may be used, provided that these columns are capable of separating at least 75 mg of residue organics with the resolution (Tabor 1984) obtainable on an analytical scale, i.e., 25 µg of sample.

6.1.3 A mercury lamp UV detector fitted with a 254 nm filter (Waters Associates Model 440 or equivalent).

6.1.4 Two pen recorder for monitoring both UV detector output and solvent composition (Fisher Scientific Recordall(a) Series 5000, Number 13-939-20 or equivalent).

6.1.5 Sample evaporator (Organomation Associates, Inc., N-Evap(a) Model 111 or equivalent).

6.1.6 Evaporative concentrator, modified micro Synder, 4 mL tube (Kontes No. K-569250 or equivalent).

6.1.7 Analytical balance, readable to 0.01 mg with a precision of ± 0.01 mg.

6.1.8 Muffle furnace, capable of sustaining 500 C, for use in glassware decontamination.

6.1.9 Solvent clarification kit (Waters Associates Number 85113 or equivalent) for use with HAWPO 4700 aqueous filters (Waters Associates Number 85117 or equivalent) and FHUP 04700 organic filters (Waters Associates Number 85118 or equivalent).

6.1.10 Sample enrichment and purification cartridges, packed with octadecylsilane (Waters Associates SEP-PAK Number 51910 or equivalent).

6.1.11 Guard column (Waters Associates Number 84550 or equivalent) packed with octadecylsilane (Waters Associates Number 27248 or equivalent) for reverse phase separations or with silica (Waters Associates Number 27245 or equivalent) for normal phase separations.

6.1.12 Sonication - A sonicator capable of holding two or more 1-L solvent containers for solvent degassing (Branson Model B-32 or equivalent).

6.1.13 Evaporation and sample storage gas, nitrogen or helium, water compressed, high-purity grade.

6.1.14 Sample storage vials (Wheaton "500" amber serum bottles Nos. 223778, 223779, 223785 or 223787 or equivalent), sealable with Teflon-faced septa (Wheaton No. 224167 or 224172 or equivalent).

6.2 Cleaning of Apparatus and Glassware--

6.2.1 HPLC unit is cleaned and maintained routinely according to the recommendation of the manufacturer. This includes cleaning of in-line solvent filters, cleaning of UV detector cell and routine cleaning of the guard column.

6.2.2 Sample vials - Detergent wash, rinse with tap and ASTM Type I water, followed by drying/muffling overnight at 500 C.

6.2.3 Septum - Detergent wash, rinse with tap and ASTM Type I water followed by successive rinses with pesticide-grade acetone and hexane.

6.2.4 General glassware - clean as recommended in 44 FR 69464, December 3, 1979.

7.0 Reagents

7.1 Solvents - Acetonitrile, hexane, methylene chloride and methanol, pesticide grade or equivalent, DMSO, and pyridine, reagent grade or equivalent, stored in original containers and used as received.

7.2 ASTM Type I water, generated by a Continental/Millipore Water Conditioning system or equivalent.

8.0 HPLC Separation of Residue Organics

8.1 Solvent and Sample Preparation--

8.1.1 Solvent preparation-- All HPLC solvents are filtered and degassed prior to use as chromatographic mobile phases. The solvent is filtered, under a vacuum, through a 0.2-μ filter, Section 6.1.9, then the filtrate receiving flask is placed into the sonicator. Following 10 min of sonication, the flask is removed and the HPLC solvent line is inserted into the flask for use of the mobile phase in the chromatographic separation. Solvents are filtered and degassed daily before use. Additional information on solvent preparation for HPLC use has been described by Dolan (1984).

8.1.2 Sample preparation-- Residue organics are dissolved in the appropriate solvent according to the following procedure. For reverse phase separations, the sample is dissolved in a minimum volume of acetonitrile. The solution is diluted with water until a volume equal to the acetonitrile is added or until the solution becomes cloudy. In the latter case, additional acetonitrile is added, about 10% of the total volume or until the solution becomes clear again. For normal phase separations, the sample is dissolved in a minimum volume of methylene chloride. By adding hexane, the solution is diluted using the same method described for acetonitrile solutions.

8.2 Analytical Scale HPLC Separations--

8.2.1 Reverse phase HPLC separations--A preliminary analytical scale HPLC separation (Tabor et al. 1980, 1984, Tabor and Loper 1980, 1984, Loper et al. 1983) of an aliquot of the residue organics is conducted, using a reverse phase mode, in order to characterize the sample of residue organics. The separation is accomplished by injecting 25 µL of a 1 µg per µL solution of the residue organics into the HPLC unit operating under the following conditions; flow rate, 2.0 mL/min; initial mobile phase, water; detector setting of 0.1 absorbance units full scale. Following injection, the column is washed with water for 5 min or until no more UV absorbing, i.e. peaks >5% full scale, components are eluted. After the water wash, a linear mobile phase gradient of 45 min duration is initiated from 100% water to 100% acetonitrile. On completion of the gradient, the column is washed with acetonitrile for 10 min or until no more UV absorbing, i.e. peaks >5% full scale, components are eluted.

After completion of this HPLC run, the system is re-equilibrated to the initial conditions by using a 10 min linear gradient from 100% acetonitrile to 100% water, then flushing the system for 15 min with water before injecting the next sample.

Results from this HPLC separation will indicate the composition of the sample of the residue organics. If the majority, >75%, of the sample elutes at a solvent composition of 80% water:20% acetonitrile or later, then proceed to the preparative scale reverse phase HPLC separation of the sample to collect fractions for mutagenesis testing. If the majority, >75%, of the sample elutes before a solvent composition of 80% water:20% acetonitrile, then proceed to the analytical scale normal phase HPLC separation.

8.2.2 Normal phase HPLC separation--A preliminary analytical scale normal phase HPLC separation (Tabor and Loper 1984b, Tabor et al. 1984) of an aliquot of the residue organics is conducted if the reverse phase HPLC separation is not suitable, as described above. The separation is accomplished by injecting 25 µL of a 1 µg per µL solution of residue organics into the HPLC unit operating under the following correlations: Flow rate, 2.0 mL/min; initial mobile phase, hexane; detector setting of 0.1 absorbance units full scale. Following injection, the column is washed with hexane for 5 min or until no more UV absorbing, i.e. peaks >5% full scale, components are eluted. After the hexane wash, a linear mobile phase gradient of 30 min duration is initiated from 100% hexane to 100% methylene chloride. On completion of the gradient, the column is washed with methylene chloride for 10 min or until no more UV absorbing, i.e. peaks >5% full scale, components are eluted. Following the methylene chloride wash, a linear mobile phase gradient of 30 min duration is initiated from 100% methylene chloride to 100% methanol. On completion of this second gradient, the column is washed with methanol for 10 min or until no more UV absorbing, i.e. peaks >5% full scale, components are eluted.

After completion of this HPLC run, the system is re-equilibrated to the initial conditions as follows: A 10 min linear gradient from 100% methanol to 100% methylene chloride; a 3 min wash with methylene chloride; a 10 min linear gradient from 100% methylene chloride to 100% hexane; a 15 min wash of the column, with 100% hexane before injecting the next sample. The results of this HPLC separation will indicate the composition of the sample of residue organics, in terms of polarity of the constituents.

8.3 Preparative Scale HPLC Separations--

Based on the results of the analytical scale HPLC separation, one of the following preparative scale HPLC separations will be utilized for the complex mixture of residue organics. The preparative scale HPLC procedures are applicable to 40 to 80 mg of residue organics per run.

8.3.1 Reverse phase HPLC separation--The HPLC unit is operated under the following conditions: Flow rate, 2.0 mL/min; detector setting of 2.0 absorbance units full scale; initial mobile phase, water. Following sample loading, the column is washed with water for 5 min or until no more UV absorbing, i.e. peaks >5% full scale, components are eluted. During this time, the eluate is collected as Fraction A. Then the eluate collection vessel is changed to a new one for the collection of Fraction B and a linear mobile phase gradient from 100% water to 75% water: 25% acetonitrile is run over a period of 5 min. The column is washed with this solvent composition until no more UV absorbing, i.e. peaks >5% full scale, components are eluted. During this time, the eluate continues to be collected as Fraction B. Then the eluate collection vessel is changed to a new one for the collection of Fraction C, and a linear mobile phase gradient is run over a period of 5 min to a solvent composition of 50% water:50% acetonitrile. The column is washed until no more UV absorbing, i.e., peaks >5% full scale, components are eluted; the collection of Fraction C continues during the wash. The eluate collection vessel then is changed to a new one for the collection of Fraction D, and a linear mobile phase gradient is run over a period of 5 min to a solvent composition of 25% water:50% acetonitrile. Again, the same procedure is followed, i.e. column elution followed by changing of the collection vessel for Fraction E. A linear mobile phase gradient is run over a period of 5 min to a solvent of 100% acetonitrile. The column is washed until no more UV absorbing, i.e., peaks >5% full acale, components are eluted.

The five Fractions, A-E, are processed immediately, as described below, for bioassay.

8.3.2 Normal phase HPLC separations--For those samples of residue organics better suited for separation via normal phase HPLC (see Section 8.2.2), the following procedure is followed: Flow rate, 2.0 mL/min; detector setting of 2.0 absorbance units full scale; initial mobile phase, hexane. Following sample loading, the column is washed with hexane for 5 min or until no more UV absorbing (peaks >5% full scale), components are eluted. During this time, the eluate is collected as Fraction A. The eluate collection vessel is changed to a new one for the collection of Fraction B, and a linear mobile phase gradient from 100% hexane to 65% hexane:35% methylene chloride is run over a period of 5 min. The column is washed with this solvent composition until no more UV absorbing (peaks >5% full scale), components are eluted. During this time, the eluate continues to be collected as Fraction B. Then the eluate collection vessel is changed to a new one for the collection of Fraction C, and a linear mobile phase gradient is run over a period of 5 min to a solvent composition of 35% hexane:65% methylene chloride. The column is washed until no more UV absorbing i.e., peaks >5% full scale, components are eluted; the collection of Fraction C continues during the wash.

The eluate collection vessel then is changed for the collection of Fraction D, and a linear mobile phase gradient is run over a period of 5 min to a solvent of 100% methylene chloride. The column is washed until no more UV absorbing components are eluted; the collection of Fraction D continues during the wash. Change the collection vessel for the collection of Fraction E and run a linear mobile phase gradient over a period of 5 min from 100% methylene chloride to a solvent composition of 65% methylene chloride: 35% methanol. The same procedure is followed: Column washing until the UV absorbance is <5% full scale followed by changing of the collection vessel to a new one for the collection of Fraction F. A 5 min linear mobile phase gradient is then run to a solvent composition of 35% methylene chloride:65% methanol. Again the same sequence is followed, then the collection vessel is changed for Fraction G. A 5 min linear mobile phase gradient is then run to a solvent of 100% methanol. The column is washed with methanol until no more UV absorbing (i.e., >5% full scale), components are eluted.

The seven Fractions, A-G, are processed immediately as described below, for bioassay.

8.4 Preparation of HPLC Subfractions Residue Organics for Bioassay--

Two procedures can be used to remove the HPLC solvents from the subfractions prior to testing the residue organics for mutagenic activity. Both methods have been used in situations similar to those employing the protocols in this document, and each is described below.

8.4.1 Evaporation of the solvent--Gentle evaporation of the solvent has been employed for those fractions collected from normal phase HPLC separations (Tabor et al. 1984, Tabor and Loper 1984b). The volume of each HPLC subfraction is reduced using the micro-Snyder apparatus. Usually a measured aliquot of an extract is concentrated at one time, rather than concentrating the whole extract. The sample is gently heated using the N-Evap bath. As the solution is concentrated, some constituents may come out of the solution. If so, a small volume of acetone may be added to keep the components in solution until sufficient evaporation has occurred to azeotrope the remaining HPLC solvent from the subfraction. Usually three to four additions of acetone are required. Final volume of the acetone concentrates are recorded and these samples are stored in Teflon-capped amber vials at -20 C until mutagenesis testing. At the time of bioassay, an aliquot of the residue solution is removed from the sample vial; typically this aliquot is adjusted to the necessary bioassay volume with DMSO. If it is necessary to know the mass of the residue organics per dose, the amount of organics should be determined gravimetrically on a separate aliquot of the acetone solution of residue organics.

8.4.2 Solvent exchange--Small chromatographic columns of octadecylsilane (C-18 SEP-PAK cartridges) have been used for those fractions collected from reverse phase HPLC separation (Tabor and Loper 1980, Tabor et al. 1980). In this procedure, HPLC subfractions B-E are diluted with two volumes of water and this solution is passed through a C-18 SEP-PAK previously activated as follows: Slowly pass 10 mL of acetonitrile through the SEP-PAK, according to the instructions from the manufacturer; the SEP-PAK is then washed with 20 mL of Type I water. Following the slow passage of the HPLC subfraction through the SEP-PAK, 5 mL of air is gently passed through the SEP-PAK to remove the residual solvent. The residue organics are eluted from the SEP-PAK by the slow passage of 5 mL methylene chloride through the cartridge. The volumes of the methylene chloride concentrates are recorded, and these samples are stored in Teflon-capped amber vials at -20 C until mutagenesis testing. These solutions are then processed in the same manner as the acetone concentrates in Section 8.4.1 for the preparation of DMSO solutions for bioassay.

The aqueous wash, Fraction A, from the reverse phase HPLC separation is processed according to the evaporation procedure in Section 8.4.1.

9.0 Blanks and Controls

9.1 Solvent Blanks--

Samples of each lot of hexane, methylene chloride and methanol are concentrated for mutagenesis testing. These solvents are processed according to the procedure in Section 9.1 of the Drinking Water Protocol (Section 3.0 of Proceedings).

9.2 HPLC System Blanks--

9.2.1 Analytical scale HPLC blanks--A complete chromatogram is run for both normal phase and reverse phase separations by injecting the sample solvent into the system. If peaks >10% full scale, 0.1 absorbance units, are observed, the columns should be cleaned according to the recommendations of the manufacture as outlined below.

9.2.1.1 Cleaning of reverse phase HPLC columns--Using methanol as a mobile phase, flowing at 1.0 mL/min, 200 µL of DMSO is repeatedly injected three to five times followed by a 100 mL solvent flush with methanol.

If the DMSO procedure does not clean the column adequately, determined by repeating an analytical solvent blank run, the system is washed at 2 mL/min for 5 min with 100% methanol followed by linear mobile phase gradient to 100% methylene chloride over a 15 min time period. The system is washed for 10 min with methylene chloride then re-equilibrated to 100% methanol by a 5 min linear mobile phase gradient from methylene chloride to methanol.

9.2.1.2. Cleaning of normal phase HPLC columns--Using hexane as a mobile phase, flowing at 1.0 mL/min, 200 µL of pyridine is injected three to five times followed by a 100 mL solvent flush with hexane. The cleaning is repeated if an analytical solvent blank run does not meet specifications. If the second cleaning procedure does not restore the column performance adequately, the HPLC column is replaced.

9.2.2 Preparative scale HPLC blanks--Following a preparative scale HPLC run, normal or reverse phase, the appropriate analytical scale solvent blank run is performed. If the column needs cleaning, the procedure in Section 9.2.1 is followed.

10.0 Sample Storage

Acetone and methylene chloride concentrates of the HPLC subfractions of residue organics are stored in Teflon-capped amber vials, containing an inert gas (nitrogen or helium) atmosphere, at -20 C until mutagenesis testing or further chemical analysis/HPLC separation. The initial mutagenicity testing should be conducted within two weeks since it has been noted that the bioactivity of some residue organics decreases with time (Loper and Tabor unpublished).

11.0 Data Records

Records to be maintained on the sample include a description of the residue organics sample to be separated, information on the preparation of the residue organics, mutagenesis data for the initial testing of these samples and chain of custody forms for each sample. Additionally, records to be maintained on the HPLC separation include HPLC system performance data, chromatograms on both analytical and preparative separations, volumes of HPLC subfractions collected, data on quality control of columns and solvents, volumes of concentrated HPLC subfraction extracts and storage data and any unusual occurrences during the HPLC separations. Chain of custody forms should be executed for each sample from the HPLC procedure through the mutagenesis testing step.

Records needed to be maintained for the columns include each lot of SEP-PAK cartridges, each lot of solvents used and the overall maintanence/system performance of the HPLC system.

12.0 Calculations

From the data records, the final volume and/or weight of each HPLC subfraction is related directly back to the sample of residue organics and also to the original environmental or waste sample used in the preparation of the residue organics. Therefore, it is imperative that accurate records of all weights/volumes be maintained throughout all operations.

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